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Characterization of a cytoplasmic protein localized to the bile canaliculi of hepatocytes

By

Bin Li

A thesis submitted to the Faculty of Graduate Studies and Research in partial Fulfillment of the requirements for the degree of

Master of Science

In

Molecular Biology and Genetics

Department of Biological Sciences
Edmonton, Alberta
Spring, 2001

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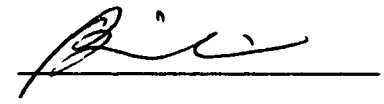

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Characterization of a cytoplasmic protein localized to the bile canaliculi of hepatocytes" by Bin Li in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology and Genetics.

Dr. Warren J. Gallin

Dr. John Bell

Dr. Luis Agellon

Date: Dec. 22, 2000

This thesis is dedicated to my parents, my wife and my son who will be born in a couple of weeks.

Abstract

Polarization of epithelial cells is an important process during the development of metazoans. Polarized epithelia are essential in many different physiological activities. The development of the bile canaliculus network is such an event, which depends on the normal polarization of hepatocytes. During the process of polarization, a subset of cellular proteins is specifically localized to the apical surface of the hepatocyte, which forms the boundary of the canaliculus. We screened a chicken liver cDNA library with antibodies against canalicular markers and isolated a full-length cDNA 9C5. The sequence predicts 9C5 is a zinc finger protein with multiple leucine zipper domains. A BLAST search showed that 9C5 is possibly the chicken homologue of a human protein FIP2, which is involved in apoptosis. We constructed two VSV-G tagged 9C5 plasmids and successfully expressed them in Cos7, MDCK and chicken liver cells. The data suggest that 9C5 may function as part of the subcanalicular complex during the polarization of hepatocytes and development of the bile canaliculus.

Acknowledgements

First and foremost, I would like to express my gratitude to my supervisor, Dr. Warren Gallin, for his encouragement, enthusiasm and help throughout the course of this project. I would also like to thank Dr. John Bell and Dr. Luis Agellon for being in my supervisory committee and for their invaluable suggestions.

It's been a pleasure to be working with all the members in Dr. Warren Gallin's lab, who have always given me hands whenever I need them. I would also like to express my thanks to Mr. Rakesh Bhatnagar. I could not have finished all my fluorescence pictures without his help.

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List of Abbreviations

GPI	Glycosyl-phosphatidylinositol
ECM	Extracellular matrix
MDCK	Madin-Darby canine kidney
ATPase	Adenosine triphosphatase
PTP	protein tyrosine phosphatase
GTP	Guanosine triphosphate
TGN	Trans-Golgi network
VIP21	Vesicle interacting protein 21
Tin-2	Tail interacting protein-2
DPPIV	Dipeptidyl-peptidase IV
HNF	Hepatic nuclear factor
TNF	Tumor necrosis factor
FIP	14.7K interacting protein
DMEM	Dulbecco's modified Eagle's medium
FCS	Fetal calf serum
S-MEM	Minimum essential medium
PEI	Polyethylenimine
ORF	Open reading frame
UTR	Untranslated region
RIP	Receptor interacting protein
NIK	NF-κB inducing kinase
IKK	IκB kinase

Introduction

General Background

The formation of a polarized epithelium from unpolarized precursors is a fundamental process in the development of multicellular animals. Its most striking characteristic is the differentiation of structurally and functionally distinct apical and basal-lateral domains (Simons and Fuller, 1985). The apical domain, usually covered by microvilli, is involved in regulating the uptake and secretion of ions and molecules and helps maintain concentration gradients between the compartments that epithelial cell layers separate (Simons and Fuller, 1985). The glycolipids (van Meer et al., 1987) and glycosyl-phosphatidylinositol (GPI) linked proteins (Simons and van Meer, 1988) are normally found in the apical surfaces.

The lateral membrane of epithelial cells, on the other hand, directly contacts the neighboring cells and thus it is specialized for cell-cell adhesion and cell-cell communication. The lateral domains are rich in cell-cell adhesion molecules and junctional complexes, which all contribute to interconnecting cells with each other. Gap junctions, *adherens* junctions, and desmosomes all belong to this group (Simons and Fuller, 1985).

Attached to a basement membrane, the basal surfaces of cells are where the cell-substratum interactions occur. Because it is close to the vascular supply, the basal surface

is also involved in exchanging information with other parts of the body through the circulatory system. Basal and lateral surfaces are often grouped together as the basal-lateral domain due to the fact that they share many common components, including common proteins and a similar phospholipid composition (Simons and Fuller, 1985). The tight junction forms a physical barrier and structural boundary between the apical and basolateral domains, and becomes an indispensable structure during the polarization of epithelial cells (Simons and Fuller, 1985). In fact, most proteins in the basolateral domain diffuse freely within the domain but they can't migrate into the apical domain.

Polarization is necessary for various aspects of the normal physiological functioning of different organisms, including vectorial transport of ions and solutes across cell layers, activation of the immune reaction in response to chemotoxins, and positional cell movement (Drubin and Nelson, 1996). Therefore, defining the mechanisms that control polarization is critical not only to understanding the differentiation process itself but also to understanding the complicated physiological functions of mature epithelial organs.

Development of polarity in cultured epithelial cells occurs in several steps. Firstly, an extrinsic spatial cue, possibly provided by the cell-cell and cell-ECM contacts, is required to mark the plasma membrane and trigger the molecular cascade that initiates polarization (Ojakian and Schwimmer, 1994; Rodriguez-Boulan and Nelson, 1989). The positional information then signals the intracellular component to assemble a localized

cytoskeletal network, which in turn directs the vesicle sorting machinery to deliver the targeted plasma proteins to their appropriate destinations (Yeaman et al., 1999). Both the locally assembled cytoskeletal structures and the resulted positional protein trafficking reinforce and strength the positional information given by the spatial cues and complete the hierarchical model for setting up cell polarity (Drubin and Nelson, 1996). Protein sorting from intracellular compartments and selective retention in the membrane will maintain the distinct distribution of membrane proteins and keep cells in polarized state (Rodriguez-Boulan and Nelson, 1989).

In a three-dimensional environment, epithelial cell polarity seems to develop after the aggregation of non-polarized cells and establishment of extensive cell-cell contacts (Ekblom et al., 1986; Fleming and Johnson, 1988). Studies in monolayers of Madin-Darby canine kidney (MDCK) cells *in vitro* clearly implicate cell-cell adhesion in the polarization process (McNeill et al., 1990; Rodriguez-Boulan et al., 1983; Vega-Salas et al., 1987). Epithelial cell-cell adhesion is mediated mainly by E-cadherin, a member of the Ca2+-dependent cadherin superfamily (Kemler, 1992). In suspension culture, MDCK cells form aggregates, dependent on E-cadherin-mediated cell-cell adhesion, and then rapidly develop polarity (Wang et al., 1990). On the other hand, the addition of E-cadherin specific antibody can lead to loss of cell polarity in polarized cells (Behrens et al., 1985). Therefore, E-cadherin mediated cell-cell contact is sufficient to initiate the segregation of apical and basolateral marker proteins to distinct regions of the membranes

(Wang et al., 1990). More tellingly, the ectopic expression of E-cadherin in transfected, normally unpolarized, fibroblasts induces accumulation of Na+, K+-ATPase to the sites of E-cadherin-mediated cell-cell contacts, similar to that in polarized epithelial cells. The expression of E-cadherin with a truncated cytoplasmic domain however does not (McNeill et al., 1990). This strongly indicates a direct role for E-cadherin as an inducer of cell polarity.

Integrins mediate most cell-ECM adhesion (Clark and Brugge, 1995), which is also important in generating membrane asymmetry. While cell-cell contact triggers the segregation of apical surface proteins to the free cell surface (McNeill et al., 1990), the absence of ECM causes the tight junction protein ZO-1 to spread along the cell-cell contact area where its distribution overlaps that of Na^+,K^+-ATPase, the basolateral destined protein (Wang et al., 1990). MDCK cell aggregates from suspension culture that are polarized solely by cell-cell adhesion, lose polarity and rapidly reform it in the reversed direction when they are placed in a collagen gel (Wang et al., 1990). In other words, the interaction with ECM leads to reversal of the axis of cell polarity. The reversal of polarity is disabled when monoclonal antibody against the β1 integrin subunits is utilized in the experiment (Ojakian and Schwimmer, 1994). Cell-ECM adhesion is therefore particularly of importance in orientating the apicobasal axis of polarity. Taken together, cell-cell adhesion is sufficient to induce the segregation of plasma membrane

proteins to distinct domains. Cell-ECM adhesion further provides the direction for setting up the axis of polarity.

E-cadherin mediated cell-cell adhesion and integrin mediated cell-ECM adhesion can initiate the polarization and provide spatial cues to accomplish a fully developed polarization. However, the mechanism by which the spatial cue is propagated to the rest of the cell surface and the inside of cells is still unclear. Both cadherin and integrin-mediated adhesions induce localized assembly of specialized cytoskeletal and signaling networks where the contacts occur (Clark and Brugge, 1995; Kemler, 1992). These assemblies are likely responsible for directing the spatial organization of plasma membrane proteins, signaling molecules, microtubules, and the secretory machinery locally and globally.

The cell-cell adhesion molecule E-cadherin forms a complex with catenins (Gumbiner, 1996), which is required for cadherin-mediated cell adhesion (Nagafuchi and Takeichi, 1988). α-catenin co-localizes extensively with E-cadherin in fibroblasts and has actin-binding activity (Knudsen et al., 1995), so it probably functions to link the cadherins to the actin cytoskeleton (Rimm et al., 1995). β-catenin mediates the attachment of E-cadherin to α-catenin (Rimm et al., 1995). The complex formed between cadherin-catenins and the actin cytoskeleton potentially provides a protein scaffold that may be important in recruiting the signaling molecules (Yeaman et al., 1999).

Tyrosine phosphorylation is suggested to be involved in regulation of the cadherin-catenin complex. Expression of the v-src gene causes rapid loss of epithelial polarization in MDCK cells and the dedifferentiation effect correlates with tyrosine phosphorylation of the β-catenin (Behrens et al., 1993). Receptor protein tyrosine phosphatase PTPμ (protein tyrosine phosphotase μ) is co-immunoprecipitated with cadherin and catenin and it also binds directly to E-cadherin *in vitro* (Brady-Kalnay et al., 1995). These observations all indicate the involvement of phosphorylation in the function of the cadherin-catenin complex, the assembly of cytoskeleton and signal transduction. Other signaling pathways, such as RAS and small GTP-binding (Guanosine triphosphate-binding) proteins, may also associate with the locally assembled cadherin-catenin-cytoskeleton complex (Lewis et al., 1994). Recently, it was found that Cdc42 and Rac1 are required to stabilize the cadherin-catenin complex (Kuroda et al., 1998). An effector of Cdc42 and Rac1, IQGAP1, has been shown to interact with β-catenin and the cytoplasmic domain of E-cadherin and interfere with the binding of α-catenin to β-catenin (Fukata et al., 1999). It seems Cdc42 and Rac1, and IQGAP1 serve as positive and negative molecular switches of cadherin activity, respectively (Kaibuchi et al., 1999).

The actin cytoskeleton is also associated with integrin adhesion receptors. α-actinin, the cross-linker of actin, binds vinculin (Wachsstock et al., 1987), which binds talin (Burridge and Mangeat, 1984), a integrin binding protein (Horwitz et al., 1986).

Thus integrin may be linked to actin cytoskeleton through α-actinin, vinculin and talin. Similar to the local assembly of cadherin-catenin complex, the integrin-talin-vinculin complex is thought to be critical in cell-ECM adhesion and also provide a protein scaffold for a signaling network assembly (Yeaman et al., 1999). Again, the Rho family of GTPases are implicated in integrin signal transduction to regulate cell polarity, motility, and many other cellular events (Clark and Brugge, 1995). Integrin-mediated adhesion can stimulate Rho family proteins and activation of Rho GTPases would in fact enhance integrin function and downstream signal transduction (Giancotti and Ruoslahti, 1999).

Both cadherin-mediated cell-cell and integrin mediated cell-ECM adhesion can cause formation of a local cytoskeleton complex which provides a framework to incorporate signaling molecules and relay the polarization signal to other parts of cells. The assembly of submembrane cytoskeleton networks at the site of cell-cell or cell-ECM contacts has proved to be critical in the formation of basolateral membrane domains (Hammerton et al., 1991; Hu et al., 1995; Marrs et al., 1995; McNeill et al., 1990). The interaction between E-cadherin and catenin is shown to be sufficient and required for localizing Na^+-K^+-ATPase to the cell-cell contacting area (McNeill et al., 1990). The assembly of a cytoskeleton complex under cell-cell or cell-ECM contacting surface is likely the first step in propagating polarization signals from the extrinsic cue, and it serves to turn on the global change of the polarizing cells.

Under the noncontacting (apical) surfaces, the membrane associated actin cytoskeleton network has also been well characterized (Mooseker, 1985). Villin and fimbrin play direct roles in organizing the actin filaments, which are the central core of each microvillus (Bretscher, 1981; Bretscher and Weber, 1980). Although the apical surfaces of cells do not contact any cells or ECM, it is still possible that spatial signals from cell adhesions on the basolateral domain are transduced through the cytoskeleton network to direct the polarization process at the apical surfaces. Active cytoskeleton reorganization has been observed under the free cell surface (Mooseker, 1985). Villin is found near the sites of cell-cell contacts before the formation of apical microvilli, but it becomes limited to the apical cortex soon after (Ezzell et al., 1992). Again, villin could be directed by a polarization signal to migrate to the apical area through the cytoskeletal complex, and villin has been implicated in linking the apical membrane to the cytoskeleton structures (Bergson et al., 1993).

Cell adhesion also causes the reorganization of microtubules. Originating from perinuclear region of the cytoplasm in nonpolarized cells, microtubules accumulate under the apical surfaces and orient along the apicobasal axis of the polarized cells (Bacallao et al., 1989). With the redistribution of microtubules, the compartments related to protein sorting machinery are also coincidentally localized to the apical and basal cytoplasm (Bloom and Goldstein, 1998). Microtubule motors such as dynein or kinesin, may facilitate relocating these compartments (Walker and Scheetz, 1993). Disruption of

microtubules delays protein delivery to the cell surface (Mays et al., 1993). The reorganization of microtubules and the protein sorting machinery may also be the result of signaling from the spatial polarization signal by cell adhesions.

The distinctive distribution of components between apical and basolateral domains tells us that protein sorting and targeting to appropriate cell surfaces is essential in polarization. It is still not clear how this process works. Sorting of apical and basolateral membrane proteins occurs in the TGN (trans-Golgi network) and endosomes (Yeaman et al., 1999). Targeting of proteins to the basolateral surface mostly recognizes cytoplasmic sorting signals (Aroeti and Mostov, 1994; Matter and Mellman, 1994). A critical tyrosine residue in the context of large hydrophobic amino acids was first identified as an important type of the basolateral signal (Eberle et al., 1991). Later it was proposed it is the tight β-turn conformation being recognized by sorting machinery (Le Gall et al., 1997). When introduced into heterologous proteins, these motifs can divert an otherwise apical protein to the basolateral membrane (Matter and Mellman, 1994).

The apical sorting pathway seems to have a fundamentally different transport mechanism than the basolateral delivery. Sphingolipid-cholesterol rafts are suggested to be used to transport sorted apical membrane proteins, in the exoplasmic leaflet of the TGN (Simons and Ikonen, 1997). The glycosylphosphatidyl inositol (GPI)-anchor may mediate the clustering of the apical rafts and act as an apical sorting signal (Brown et al., 1989). Disruption of the sphingolipid-cholesterol rafts by depleting cells of cholesterol

causes missorting of some apical proteins (Keller and Simons, 1998). N-glycans have also been demonstrated to behave as apical sorting determinants in the raft-dependent route (Scheiffele et al., 1995). While many apical signals use a raft to transport apical proteins, some proteins may use a raft-independent pathway to reach the apical surface (Mays et al., 1995). Basal-lateral sorting signals are located in the cytoplasmic domain, but apical sorting signals appear to reside in the extracellular domain, although their identity is much less clear (Keller and Simons, 1998).

After apical and basolateral membrane proteins are sorted in the TGN, they are packaged into different transport vesicles to be shipped to their destinations. VIP21/caveolin has been suggested to be necessary for recruitment of GPI-proteins into the apical rafts (Zurzolo et al., 1994). VIP21/caveolin is a cholesterol-binding protein (Murata et al., 1995) and it is present on post-Golgi transport vesicles (Scheiffele et al., 1998). Anti-caveolin-1 antibodies decrease the delivery of some apical proteins to the apical membrane (Scheiffele et al., 1998). The factors that mediate basolateral sorting have yet to be defined. Tin-2 (Pimplikar et al., 1994) and p200/myosinII (Musch et al., 1996) are both involved in the basolateral transport vesicles and absent from apical transport vesicles. Nevertheless, they are the candidates that participate in delivering the sorted membrane proteins to their destined membrane domains and finishing the polarization process (Figure 1).

Polarization is involved in many physiological activities. In liver, all liver parenchymal cells are polarized, which is crucial to the development of bile canaliculi and many functions of liver.

The Development of Bile Canaliculi

The liver parenchyma consists of polarized hepatocytes. The bile canaliculus is the space surrounded by the apical surfaces of adjacent hepatocytes. The bile canaliculi are continuously interconnected and well-coordinated to form a three-dimensional network, which is the intercellular secretory system that carries the bile from the hepatocytes to the bile duct. As with other epithelial cells, the bile canalicular (apical) surfaces are enriched in microvilli. A distinct set of proteins are located in the bile canalicular surface (Rodriguez-Boulan and Nelson, 1989; Simons and Fuller, 1985), such as leucine aminopeptidase (Roman and Hubbard, 1984b), B10 antigen (Maurice et al., 1994) and 9B2 antigen (Chiu et al., 1990). Separated from the canalicular membrane by tight junctions, the basal-lateral domains face the sinusoidal spaces and communicate with other cells.

Many factors are involved in the polarization/differentiation of hepatocytes. Cell-cell and cell-matrix interaction, cell shape, vascularization of the liver tissue, and soluble factors may all play roles (Ben-Ze'ev, 1991; Ingber, 1991; Musat et al., 1993). As in permanent polarized cell lines, E-cadherin mediated membrane contact is critical to the

formation of intact bile canaliculi and anti-E-cadherin antibodies cause a disruption of long-range networks of bile canaliculus (Terry and Gallin, 1994). Cell-ECM interaction has also been reported to help in developing hepatocyte differentiation (Ben-Ze'ev, 1991).

Cytoskeletal structures are implicated in the formation of bile canaliculi. An elaborate cytoskeletal network exists under the bile canalicular domain. It consists of actin microfilaments and cytokeratin intermediate filaments. The actin structure includes the pericanalicular web and the microvillus core filaments. The pericanalicular sheath consists of cytokeratin intermediate filaments, and it has been thought to provide a scaffold for the development of the bile canaliculus (Ohta et al., 1988). The variation of the spatial distribution of intermediate filaments observed during different stages of liver differentiation reflects the involvement of cytoskeletal structures in the polarization of hepatocytes (Vassy et al., 1997). Canaliculus formation was demonstrated to be accompanied by a distinct accumulation of cytoskeletal and microvillar proteins at the apical surfaces (Sormunen et al., 1997), indicating their roles in the development of bile canaliculus network.

In hepatocytes, apical proteins are initially transported to the basolateral membrane and then they are internalized and sorted through transcytotic carrier vesicles to the apical domain (Hubbard, 1991), although a direct route for the apical targeting in hepatocytes has been suggested (Ali and Evans, 1990). Therefore, vesicle transcytosis, which may be the only way an apical protein can reach the apical membrane, is very

important in hepatocytes. When dipeptidyl-peptidase IV (DPPIV), an apical protein in hepatocytes, is expressed in MDCK cells, it arrives at the apical membrane via transcytosis (Casanova et al., 1991). But if a truncated soluble form of DPPIV is used, most of it is transported directly to the apical membrane (Weisz et al., 1992). This suggests there are two signals on apical proteins in hepatocytes, an apical signal and a competing basolateral signal in the cytoplasmic domain.

The movement of apical destined vesicles likely involves both microtubule and actin-associated motors (Durand-Schneider et al., 1987; Phillips et al., 1975). Microtubules extend throughout the hepatocyte between the basolateral and apical domains (Perez et al., 1998). The disruption of microtubules inhibits the delivery of apical proteins (Wilton et al., 1997). Both actin microfilaments and intermediate filaments in hepatocytes are suggested to play essential roles in cell stability, intracellular organization, transport of vesicles and bile (Lazarides, 1980; Phillips et al., 1975). However, the polarized membrane traffic in hepatocytes has yet to be fully characterized.

A number of transcription factors have also been implicated in regulating liver development. HNF3 and HNF4 are both required for the complete differentiation of hepatocytes (Duncan, 2000). Regulation of gene expression by NF-κB signaling pathway and its antiapoptotic function have both been involved in the development of liver (Beg et al., 1995; Li et al., 1999a). The differentiation of hepatocytes and the morphogenetic polarization process are thought to depend on the coordinated actions of multiple

transcription factors (Duncan, 2000), although the detailed picture is not known yet. Recently, NF-κB has been shown to specifically protect healthy differentiating hepatocytes from TNF-α induced apoptosis (Li et al., 1999c; Rosenfeld et al., 2000). In other words, the differentiation and polarization of hepatocytes are supervised by NF-κB regulation. Therefore, a link exists between the development and differentiation of hepatocytes and the regulation of NF-κB and other transcription factors.

The asymmetric distribution of cell surface components of polarized hepatocytes allows us to study the polarization of hepatocytes and the formation of bile canaliculi with the help of monoclonal antibodies (Herzlinger and Ojakian, 1984; Roman and Hubbard, 1984a; Roman and Hubbard, 1984b). In fact, early investigations on the development of bile canaliculi largely focused on the identification of the specialized domains of hepatocytes and the characterization of markers specific for these domains (Cook et al., 1983; Roman and Hubbard, 1984a; Roman and Hubbard, 1984b). Production of monoclonal antibodies has allowed identification of a number of proteins that are expressed specifically on the bile canalicular surface of hepatocytes. To understand how these bile canalicular proteins are expressed on the apical surface is of fundamental importance to learning how the normal bile canaliculus network is developed.

In rats, studies have been focused on 14 day or older embryos (Hubbard et al., 1985; Maurice et al., 1994). The polarized distribution of membrane proteins has been established before day 14 of gestation, indicating that initiation of polarization of hepatocytes starts very early in the development (Feracci et al., 1987). To define this process step by step, it is necessary to study earlier embryos. It is difficult to do so in rats because numerous hemopoietic cells exist and the hepatocytes are extremely distorted at that time (Feracci et al., 1987). This is not the case in chickens. Although the hepatocytes are organized as a set of anastomosing tubes in the chicken embryo instead of a one cell thick plate in rats, the development of an individual hepatocyte and the ultrastructure of the canaliculus is still comparable (Gallin and Sanders, 1992).

A set of monoclonal antibodies that can specifically recognize the bile canalicular surface of hepatocytes in chicken was developed and used to obtain information about the development of the canaliculus network in chicken (Gallin and Sanders, 1992). Electron microscopy confirms the specificity of the five antibodies developed for bile canaliculi in chicken embryos. Immunogold staining shows the expression of these bile canalicular marker antigens is either at the luminal cell surfaces or located subluminally (Gallin and Sanders, 1992). They could be either apical proteins or proteins associated with vesicles or other structures restricted to the region of bile canaliculi. In unpolarized hepatocytes, however, these antibodies stain the cytoplasm punctately, which suggests possible association with vesicles.

Immunofluorescent staining with these antibodies in a chicken embryo first detects canaliculi in 4-day embryos. By 7 days post laying, bile canalicular marker antigens are expressed throughout the entire liver, and localized to mature and functional canaliculi (Gallin and Sanders, 1992). Electron microscopy also shows early development of a distinguishable apical surface of hepatocytes in 4-day chicken embryos. By Day 6, the structures of the bile canaliculus network have become fairly mature throughout the liver, and extensive junctions have been observed at the cell contacts adjacent to the canaliculus (Gallin and Sanders, 1992).

The development of bile canaliculi is in fact a process in which a previously homogeneous plasma membrane differentiates into distinct domains that exhibit a polarized distribution of membrane proteins. Therefore, characterizing these polarized distributed proteins and studying their biosynthesis and intracellular transport is one of the important and effective approaches to understand the development of bile canaliculi. Most of the studies to date on bile canaliculi are based on the observations of the synthesis, transport and localization of individual canalicular marker proteins in fully polarized hepatocytes (Chiu et al., 1990; Herzlinger and Ojakian, 1984; Maurice et al., 1994; Musat et al., 1993; Sormunen et al., 1997; Bartles et al., 1987).

We decided to take a similar approach to isolate one or more bile canalicular marker proteins and characterize their expression, localization and functions. We cloned the gene for a chicken bile canalicular protein that specifically reacts with one of the five

monoclonal canalicular antibodies (9C5), by screening the chicken embryo liver cDNA ZAPII expression library (Stratagene). The 9C5 coding region contains 556 amino acids with three leucine zippers and one zinc finger motif. BLAST searching shows 9C5 is very likely the chicken homologue of a human protein FIP2 (Li et al., 1998). FIP2 is indirectly involved in the apoptotic pathway and its C-terminal end was isolated by using the yeast two-hybrid system to search for the interacting partners of hungtintin, the causative protein in the Huntington's disease (Faber et al., 1998; Li et al., 1998). We successfully expressed VSV-G (van der Spek et al., 1994) tagged plasmid constructions in several permanent cell lines and liver organ culture. It localization was confirmed to be overlapping with the endogenous 9C5 protein. The data strongly suggests that 9C5 is involved in the polarization process of hepatocytes, and further study of 9C5 will provide more insight into the development of bile canaliculus network.

Materials and Methods

Cell Lines

Cos7 and MDCK cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM from GibcoBRL) supplemented with 10% fetal calf serum (FCS from GibcoBRL). Gentamycin at 10μg/ml was used. Primary chicken embryo liver cell monolayer cultures were maintained in DIOD medium ($0.2X10^{-3}$U/ml insulin, 67.4mg/liter ornithine and 10μM dexamethasone in DMEM) with 1μg/ml Gentamycin. A liver organ culture was maintained in DIOD medium.

Preparation of Liver Cell Culture

We used chicken embryos from approximately 8-10 day incubated eggs and dissected the liver into ice-cold Minimum Essential Medium (S-MEM from Gibco-BRL). The dissected livers were rinsed and swirled in 25ml prewarmed (37°C) S-MEM on a rotary shaker at 150rpm for 5 min at 37°C twice. Then livers were swirled in 10ml CTB (0.1% collegenase, 0.1% 1:250 Trypsin and 10% FCS, in 0.8% NaCl, 0.03% KCl, 0.005% NaH_2PO_4, 0.0025% KH_2PO_4, 0.1% $NaHCO_3$ and 0.2% glucose) at 150rpm for 5 min at 37°C twice, and broken up by pipetting up and down in the last CTB digestion with a wide-mouth fire-polished Pasteur pipette. The cell suspension was then transferred to a 15ml T.C. tube with 1ml FCS and the tube was topped up with S-MEM-DNase (10μg/ml DNase), and spun in a clinical centrifuge at speed 2 for 4 min to pellet the cells. The supernatant was discarded and the cells were resuspended in 10ml S-MEM-DNase. The cells were filtered through a coarse Nytex filter (80 mesh) and allowed to flow into a ice-cold tube by gravity. The tube was topped up with S-MEM-DNase and spun again.

The supernatant was again discarded and cells were resuspended in 10ml S-MEM-DNase. Then a fine Nytex filter (225 mesh) was used to filter cells into an ice-cold tube. The tube was topped up with S-MEM-DNase and spun. This supernatant was also discarded and cells were again resuspended in 10ml S-MEM-DNase. The cells were counted using a hemacytometer and diluted to $3X10^6$ cells/ml. For immunofluorescent staining, 0.5ml chicken liver suspension containing $1.5X10^6$ cells was plated onto a flame-sterilized, round, 10mm glass coverslip in a 24-well tissue culture plate.

cDNA Library Screening

A chicken liver cDNA library constructed in ZAPII vectors was obtained from Stratagene (Cat# 935402). A colony of bacteria strain BB_4 (Stratagene) was grown in LB medium with 0.2% maltose, 0.01M $MgCl_2$ and 0.01M $MgSO_4$ overnight. The bacterial suspension was spun in a clinical centrifuge at speed 7 for 10 min and the supernatant was discarded. The pellet was then resuspended in 6~7 ml 10mM $MgSO_4$, 25 µl (2000pfu/µl) phage from the cDNA library was added into 300 µl bacteria suspension and mixed by pipetting. The mixture was kept at 37°C for 25~35 min, prewarmed (47°C) top agarose LB (1% agarose in LB medium) was quickly added to the bacteria-phage, mixed, and immediately evenly spread onto prewarmed (37°C) LBM (0.2% maltose and 0.01M $MgSO_4$) plates. The plates were incubated at 37°C until plaques were visible (about 4-6 hours). Then Hybond-N (Amersham) Nylon membranes (presoaked in 10mM IPTG) were put onto the plates and left at 37°C overnight. After cooling, each membrane was marked with needles to position it to the plate. The membranes were taken off and

blocked in 5% skim milk/T-TBS (0.1% Tween20, 50mM Tris-HCl, pH 8.0 and 150mM NaCl) at 4°C overnight.

The blocking solution was removed and chicken canalicular marker antibodies (mouse 9C5, 1H6, 6A2, 5D8 and 8G7 ascites, (Gallin and Sanders, 1992)) were added (1:500 diluted in 50% blocking solution/50% T-TBS) to membranes. They were kept on the shaker at 4°C for 8 hours, then washed 5X5 min in T-TBS. The secondary antibody (Goat anti-mouse from ICN) was added (1:500 diluted in 50% blocking solution/50% T-TBS) and the membranes were still kept on a shaker at 4°C overnight, then washed 5X5 min in T-TBS. Five μCi I^{125}-protein A in 50ml T-TBS was applied and the membranes were kept on a shaker at 4°C for 4 hours, washed 5X5 min in T-TBS again, mounted on Whatman filters and taped down. They were then covered with Saran Wrap and exposed to X-ray film at -80°C for 3~4 days. The film was developed and the positive plaque plugs were stabbed out. They were soaked in SM solution (NaCl 0.1M, $MgSO_4$.7H2O 8mM, Tris pH7.5 0.5M and 0.01% gelatin) overnight. The acquired phage were diluted and screening was continued until well-isolated single positive plaques were obtained. The phagemid was rescued by *in vivo* excision following the instructions of Stratagene. Totally six independent plasmid clones were acquired. They were exposed to five individual antibody as described above they were all recognized by monoclonal antibody 9C5. All the clones were subject to automated sequencing (ABI) from both directions. One subclone and 10 internal primers were used to sequence the acquired clones (Figure 1). The sequences were compared and aligned with computer program GeneTools (Biotools). One full-length cDNA named 9C5 was assembled with these overlapped sequences.

Northern Blotting

Total RNA was extracted with Guanidinium-thiocyanate from various tissues of 10 days old chicken embryos (Ausubel et al., 1987). Every 100mg tissue was homogenized in 1ml denaturing solution (4M guanidine thiocyanate, 25mM sodium citrate, 0.5% Sarkosyl and 0.1M 2-mercaptoethanol). Sodium acetate (0.1 ml of 2M), pH4 per 100mg tissue was added and mixed. Next, 1ml water-saturated phenol and 0.2ml of 49:1 chloroform/isoamyl alcohol were added. The suspension was incubated at 4°C for 15 min and centrifuged at 10,000Xg for 20 min, at 4°C, and the upper aqueous phase was saved. Samples were mixed with one volume 100% isopropanol, incubated for 30 min at –20°C to precipitate the RNA, and then centrifuged for 10 min at 10,000g, 4°C. The supernatant was discarded. The RNA pellet was vortexed in 4M LiCl and spun down to wash out glycogen, then the RNA pellet was dissolved in denaturing solution and precipitated with one volume of 100% isopropanol for 30 min at –20°C. The RNA pellet was spun (10,000Xg, 10min) and kept, then the RNA pellet was resuspended in 75% ethanol and incubated for 15 min at room temperature to dissolve residual amounts of guanidine. This was then spun down and dried in a vacuum for 5 min. The RNA pellet was dissolved in DEPC-treated water and incubated 15 min at 55°C~60°C. A260/A280 reading ratio was used to assess the purity of the RNA preparation. Total RNA (10 μg) from each tissue was run on a formaldehyde gel and then transferred to a nitrocellulose membrane (Ausubel et al., 1987). After that the RNA was immobilized onto the membrane by baking at 80°C in a vacuum oven for 2 hours. About 600bp from the 3' end

of 9C5 cDNA (Figure 2) was labeled with [^{32}P]dCTP and Prim-a-Gene Labelling System (Promega) for hybridization.

Prehybridization was done at 65°C for 20 min in Church Hybridization Solution (6.8% SDS, 0.5M $NaHPO_4$, pH7.2, 1μM EDTA, pH 8.0 and 1% BSA) (Church and McCarthy, 1968). Then fresh prewarmed (65°C) hybridization solution, containing labeled denatured probes, was added. Hybridization was done at 65°C for 16 hours. The hybridization solution was then removed and the blot was washed twice quickly in prewarmed 65°C Wash Solution (1 μM EDTA, pH 8.0, 1% SDS and 0.04M NaHPO4, pH 7.2) to remove excess hybridization solution. Then it was washed 2X5 min at 65°C with shaking, quickly rinsed with 0.2XSSC at room temperature and air dried. The blot was wrapped with Saran Wrap and exposed to a phosphorimager.

Immunofluorescent Staining

Monolayers of Cos7 and MDCK cells were washed with warm PBS and fixed in a solution containing 3% paraformaldehyde in PBS, 0.1uM $MgCl_2$ and 0.1 μM $CaCl_2$ for 15 min at room temperature. Cells were then washed 3X3 min with PBS before they were treated with Quench buffer (50mM NH_4Cl in PBS) for 10min at room temperature. Samples were blocked and permeabilized with Triton100X/Caragennan in PBS for 30' after 3X3 min washes with PBS. Mouse 9C5 ascites and β-galactosidase monoclonal Antibody (Promega) were diluted in the blocking solution (1:1000) and incubated with samples at 4°C overnight. Samples were then washed 5x5 min with PBS and incubated overnight with Goat anti-mouse FITC conjugated secondary Antibody (ICN, 1:500 dilution in blocking solution). Samples were washed, counterstained with 1% Evan's

Blue for 5 min and mounted on glass slides with Mowiol-DABCO (Harlow and Lane, 1988). Mouse anti-VSV monoclonal antibody (1:500) and rabbit anti-VSV polycolonal antibody (1:500) were used to stain the VSV-G tagged constructs (van der Spek et al., 1994). Goat anti-rabbit Rodamine conjugated secondary Antibody (ICN, 1:500) was also used in co-staining experiments.

Whole chicken embryo liver organ cultures were fixed in a freshly made solution of 20%DMSO/Methanol overnight. The samples were washed twice with TBS for 5 min at room temperature. Samples were incubated with primary antibodies diluted in Triton100X/Carageenan/TBS (1:1000 for 9C5 antibody and β-galactosidase antibody, 1:500 for mouse VSV monoclonal antibody/rabbit VSV polyclonal antibody) overnight at 4°C, washed 5X1 hour with TBS at 4°C, and then incubated in secondary antibody (Goat anti-mouse FITC conjugated and Goat anti-rabbit Rhodamine conjugated, ICN) diluted in Triton100X/Carageenan/TBS (1:500) overnight at 4°C and washed 5X1 hour with TBS at 4°C. Mount on glass slides with Mowiol-DABCO (Harlow and Lane, 1988).

Plasmid Construction

PCR was used to remove the 5' untranslated region of 9C5 and add a VSV-G tag onto the 3' end with the following primers: forward, 5'-GG GCTAGC CACC ATG TCC AGC AAG CCG-3'; reverse, 5'-GG GAATTC TTA CTT CTT AAG TCG GTT CAT CTC TAT GTC TGT ATA ATT AAT GCA GTC. The forward primer contained a NheI restriction site and the first 15bp 3' to the start codon. The reverse primer contained an EcoRI site, a stop codon and codes for eleven amino acids of the VSV-G cytoplasmic domain immediately downstream of the terminal Asn. The 1700bp PCR product was

ligated into the expression vector pBK-CMV (Stratagene) and linarized by restriction digestion with NheI and EcoRI. To put the VSV-G tag onto 5' end of 9C5, the following primers were used: forward, GG GGATTC ACC ATGTAT ACA GAC ATA GAG ATG AAC CGA CTT AAG AAG TCC AGC AAG CCG; reverse, GG GAATTC TCA ATT AAT GCA. BamHI and EcoRI sites were used to ligate the insert into pBK-CMV. All the PCR products were sequenced to confirm.

Transient Expression of 9C5

Cells were cultured on coverslips in 24-well plates. Transfection in Cos7 cells was carried with PEI when cells reached 90% confluence (Boussif et al., 1995). One μl DNA per well was used. PEI-containing medium was changed 4-5 hrs after transfection with serum supplemented medium. Assays were started 24hrs, 48hrs and 72hrs later.

MDCK cells were transfected with a PerFect Lipid transfection kit (Invitrogen). Opti-MEM (Gibco-BRL) was used during the transfection. A 6:1 (w/w) ratio of lipid to plasmid was used in the transfection mixture (DNA 2μg/ml in Opti-MEM, lipid 12μg/ml in Opti-MEM). Complete medium was changed back after 4 hours. PFx2 (Invitrogen) gave the best result in MDCK cells.

Microprojectile bombardment (Bio-Rad) was used to transfect chicken embryo liver organ cultures. The coating and wash of microcarriers with DNA was done according to the manufacturer's instruction and $CaCl_2$ and spermidine were used (Biolistic PDS-1000/He Particle Delivery System, Bio-Rad). The chicken embryo liver organ cultures were placed on cold agarose (1%, 0.5cm thick) and bombardment was done according to instructions (Biolistic PDS-1000/He Particle Delivery System, Bio-

Rad). Gold particles with 1.0μm and 1.6μm, 25 inches Hg vaccum and 900psi rupture disks were used.

Results

Screening cDNA Library and Cloning of 9C5 cDNA

The development of the bile canaliculus network is essentially a process of polarization of hepatocytes, in which distinct apical and basolateral surfaces are formed and a specific set of canalicular proteins are expressed on or close to the apical surface of hepatocytes (Simons and Fuller, 1985). Five monoclonal antibodies that specifically recognize the bile canaliculus in hepatocytes were previously described (Gallin and Sanders, 1992). All five antibodies stained only the bile canalicular area of the hepatocytes. All of these antigens are also present in some other epithelial tissues, such as kidney and the intestine. Four are only expressed on the apical surface of cells; the 9C5 antigen appears to be seen punctately throughout the cytoplasm in epithelial cells other than hepatocytes (Gallin and Sanders, 1992). With the cocktail of the five antibodies, a chicken embryo liver cDNA library, constructed in ZAPII expression vector (Stratagene), was screened.

After screening about $1X10^6$ phage, three independent clones were isolated. Their reactions with the five individual monoclonal antibodies showed that all three clones are specifically recognized only by 9C5, one of five monoclonal antibodies (Figure 3). Continuous screening with only 9C5 Ab produced three more positive clones. The sequences indicated that five of the clones were overlapped each other and four of them have a polyA tail at the 3' end (Figure 4). The sixth one, clone b, overlaps with the other clones until basepair 1377 and then diverges into a different sequence (Figure 4). The divergent part of clone b contains a long open reading frame (ORF) that continues the ORF from the conserved N-terminal half, but the deduced peptide doesn't match any

known sequence by homology search (http://www.ncbi.nlm.nih.gov/BLAST). All the isolated clones share about 600bp sequence of the middle part of the assembled longest sequence, which indicates the epitope that is recognized by the 9C5 monoclonal antibody is encoded by this segment.

The consensus sequence assembled from these clones is 2555bp long and ends with a poly A tail. The longest ORF starts with Methionine at position 38 and extends 1668bp, encoding a 556 amino acid protein sequence. There is a 37bp 5' UTR (untranslated region) and an 847bp 3' UTR. Another Met codon is located 57bp downstream of the first Met and it could be an alternative start codon, but no consensus translational start signal is found and the sequence alignment from BLAST searching doesn't support this notion (Figure 5, 6). The full-length ORF encodes a 556 amino acid peptide and we refer to this protein product as 9C5 (Figure 6). The peptide contains three leucine zipper motifs spread along the sequence, spanning amino acids 274-295, 402-423, 464-485, and one zinc finger motif at the very C-terminal end (amino acids 534-556, Figure 6).

Several computational analyses of the obtained sequence were performed. A conserved domain search (Altschul et al., 1997) detected an ERM (Ezrin/radixin/moesin) domain (Yonemura et al., 1998). The ERM domain is shared with a group of proteins that are all located between the microvillus peripheral membrane and cytoskeletal structures and are involved in the connection of the plasma membrane to the major cytoskeletal structures (Bergson et al., 1993; Lankes and Furthmayr, 1991; Wilgenbus et al., 1993). The ERM domain spans amino acids 226-383 (Figure 4, 6). There was no nuclear localization signal (NLS) found in 9C5 amino acid sequence (http://psort.nibb.ac.jp,

(Nakai and Horton, 1999)) and it was excluded as a membrane protein because no transmembrane fragment was detected with PsortII program (http://psort.nibb.ac.jp, (Nakai and Horton, 1999)). Thus, its primary amino acid sequence predicts a cytoplasmic localization. Secondary structure analysis shows a long range of helix conformation of 9C5 (Peptool, (Wishart et al., 1994)). In a 3-D PDB database (http://www.rcsb.org/pdb, (Berman et al., 2000)) threading search, no significant hit was found.

BLAST searching shows that a human protein FIP2 shares a high degree of similarity with 9C5, with 58% identity, especially towards the C-terminal end, where two leucine zippers and the zinc finger are located (Figure 5, 6). The sequence alignment indicates that it is very likely that FIP2 is the human homologue of chicken protein 9C5. FIP2 was originally isolated in yeast two-hybrid screening and it is indirectly implicated in the apoptotic pathway. FIP2 interacts with an adenovirus protein E3 14.7-K and the interaction can reverse the antiapoptotic effect of the virus protein (Li et al., 1998). The C-terminal part of FIP2 was also picked by yeast two-hybrid as one of the interacting partners (HYPL) of Huntingtin, the protein defective in the Huntington's disease (Faber et al., 1998). The possible role of 9C5 in apoptosis and Huntingtin's disease is unclear at this stage.

Northern Blotting

Northern blotting was performed on total RNA from six different organs of chicken embryos. 9C5 mRNA is expressed in heart, muscle, brain, kidney and liver with little or no expression in lung (Figure 7). Its human homologue FIP2 is also undetectable in lung and is expressed in the other tissues at different levels (Li et al., 1998). Only one

mRNA band was observed at about 2.5-2.6kb range, although FIP2 in human has at least three major mRNA species as a result of alternative splicing in most of the tissues (Li et al., 1998). It is possible that 9C5 has more than one transcript with similar lengths that can not be distinguished in a Northern. In fact, as mentioned before, one of isolated clones (clone b) aligns with 9C5 cDNA from base 465 up to 1377, and after that it appears to become a different sequence. This could be an alternatively spliced product which has similar length with the cDNA we cloned, considering the fact that only one band shows up in a Northern (Figure 7). But it could also be a "false" ligated cDNA library product or due to recombination during cDNA library construction. However, neither extra linker sequence or splicing consensus sequence was located around the divergent point, so this issue requires further study.

Total RNA from primary liver cell cultures with different mediums was used in Land 6 (FCS-containing medium) and Lane 7 (FCS-free medium). There is no significant difference in the mRNA levels between Lane 6 and 7. So FCS does not appear to affect the transcription of 9C5.

Expression of 9C5 in Cultured Cells

The VSV-G epitope (van der Spek et al., 1994) tagged 9C5 coding region was inserted into the pBK-CMV expression vector (Figure 8). We made both 5' VSV-9C5 and 3'VSV-9C5 in order to test if the 11-amino acid VSV-G tag affects the expression or localization of the tagged constructs in transfected cells. The constructs were expressed in several cell lines including Cos7 and MDCK cells. In the nonpolarized cell line Cos7, VSV-9C5 was expressed cytoplasmically (Figure 9, 10). There is no significant

difference between the expression and localizations of 3'VSV-9C5 and that of 5'VSV-9C5 in Cos7 cells (Figure 10). Both constructs show a punctate expression pattern. In MDCK cells, a constitutively polarized cell line, unlike the endogenous 9C5 in polarized hepatocytes, both VSV-9C5 constructs are punctately expressed in the cytoplasm and no apical localization was identified (Figure 11, 12). This is not unexpected, however, due to the fact that the endogenous 9C5 is expressed cytoplasmically in epithelial cells in chicken kidney (Gallin and Sanders, 1992).

Biolistic Transfection of 9C5 into Liver Organ Culture

Using a Biolistic Particle Delivery System (Bio-Rad), VSV-9C5 constructs were transfected into 8-day chicken embryo liver organ cultures. In 8-day chicken embryos, 9C5 monoclonal antibody staining shows the characteristics of mature canaliculi and the bile canalicular marker 9C5 antigen has been expressed throughout the entire liver (Gallin and Sanders, 1992). The endogenous 9C5 staining showed an anastomosing canalicular network (Figure 13). The staining of transfected VSV-9C5 with only VSV-G antibodies showed that the constructs were successfully introduced into a few cells. Then the co-staining of endogenous and transfected 9C5 marker proteins with 9C5 antibody and transfected VSV-9C5 constructs with VSV-G antibody exhibited an overlapped, continuous network-like staining (Figure 14, 15). This result demonstrates the transfected VSV-tagged constructs are correctly expressed and localized to where the endogenous 9C5 proteins are, the bile canaliculus of polarized hepatocytes. Again, there is no significant difference between 3'VSV-9C5 and 5'VSV-9C5 constructs (Figure 14, 15). It is reasonable to suggest that the same machinery and signaling molecules direct the

expression and localization of the endogenous 9C5 proteins and the introduced VSV-9C5 constructs.

Taken together, VSV-G epitope tagged 9C5 constructs can be introduced into hepatocytes, as well as several other cells, and they are expressed and localized to the canalicular area. There is no evidence that positions of the epitope affect expression or localization of VSV-9C5 constructs.

Discussion

Using a set of monoclonal antibodies that recognize the bile canalicular marker proteins (Gallin and Sanders, 1992), we screened a chicken embryo liver expression cDNA library (Stratagene Cat# 935402). A full-length cDNA was assembled from sequences of six independent positive clones (Figure 2) that all reacted specifically with antibody 9C5 (Gallin and Sanders, 1992) and its protein product is referred as 9C5. PsortII analysis doesn't reveal any transmembrane segment or NLS in the deduced peptide sequence, so it is most likely to locate cytoplasmically (http://psort.nibb.ac.jp, (Nakai and Horton, 1999)). Previously, 9C5 antibody staining in different epithelial tissues was shown to be present only in the cytoplasm (Gallin and Sanders, 1992), which correlates with the prediction. Three leucine zippers spread along the sequence and a zinc finger motif is located in the very C-terminal end (Figure 6). Both leucine zipper and zinc finger motifs are considered important protein-protein and protein-DNA interacting motifs. They are seen in many transcription-regulatory factors. Although we haven't been able to locate 9C5 in nucleus at any time, it still remains a possibility that it may migrate into the nucleus to regulate gene expression, upon activation by upstream signals.

BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST) locates possible 9C5's human homologue protein, FIP2 (Figure 5, 6). They share 58% identity as well as the leucine zippers and zinc finger. Northern blot analysis indicates expression of both genes in different tissues with little or no expression in lung tissue. While only one band was detectable in Northern blotting for 9C5 (Figure 7), there are three major mRNA species for FIP2 with different lengths (Li et al., 1998). The C-terminal end of FIP2, which includes one leucine zipper and the zinc finger motif, is required to interact with an

adenovirus protein to indirectly activate the apoptotic pathway that is inhibited by the virus protein (Li et al., 1998). Another FIP group protein FIP3, which also shares 26% identity with 9C5 (Figure 5), has been recently shown to form homotypic dimers and trimers. The self-association domain of FIP3 covers the middle part of the protein and the second leucine zipper (Ye et al., 2000). These data all strongly indicate the leucine zipper and zinc finger motifs of 9C5 will likely mediate the protein-protein interaction between 9C5 and its interacting partners.

In polarized hepatocytes, immunofluorescence and ultrastructural immunogold studies both show that 9C5 antibody stains only the bile canalicular (apical) area of hepatocytes *in vivo* and *in vitro*, while in depolarized hepatocytes, 9C5 antigen shows a punctate cytoplasmic expression pattern (Gallin and Sanders, 1992). This suggests that 9C5 shifts to the canalicular surface or subcanalicular region during the polarization of hepatocytes while it seems to be localized punctately throughout the cytoplasm in unpolarized hepatocytes. The immunofluorescence study of the VSV-9C5 constructs further confirms the localization of 9C5 antigen during the process of polarization. In the nonpolarized cell line Cos7, the VSV-G staining of the tagged 9C5 cDNA expression is localized in a punctate pattern in the cytoplasm of cells (Figure 9, 10). In the 8-day chicken embryo liver organ culture where hepatocytes are polarized, however, VSV-9C5 staining is limited to along the well-formed bile canalicular region (Figure 11, 12, 13). These results hold true for both 3'VSV and 5'VSV tagged constructs. Therefore, localization of 9C5 antigen is regulated during the process of polarization of hepatocytes and development of the bile canaliculus network, and VSV tagged 9C5 constructs share the same characteristics with endogenous 9C5 proteins.

The bile canaliculus is the space surrounded by the apical surface of polarized hepatocytes. An elaborate cytoskeletal network exists under the bile canalicular domain (Ishii et al., 1991). It consists of actin microfilaments and cytokeratin intermediate filaments. Both actin microfilaments and intermediate filaments are suggested to play essential roles in the development of the bile canaliculus network (Lazarides, 1980; Phillips et al., 1975). In MDCK cells, the assembly of a submembrane cytoskeleton network has proved to be important in the formation of polarized membrane domains (Hammerton et al., 1991; Hu et al., 1995; Marrs et al., 1995; McNeill et al., 1990). It is thought to provide a protein scaffold to recruit structural and signaling molecules to the submembrane cytoskeletal network and direct signals to inform other parts of the cell to coordinate the polarization process. Similar subapical structures have been suggested to be required in the organization of microvilli in polarized epithelial cells (Mooseker, 1985).

The subapical localization of 9C5 antigen in polarized hepatocytes suggests 9C5 could be one of the structural proteins to recruit other components of the subcanalicular signaling network. The sequence analysis of 9C5 indicates it is possibly a protein-interacting molecule. Leucine zipper and zinc finger domains are normally thought to be involved in protein-protein and protein-DNA interaction, respectively. Its strong protein interaction potential is demonstrated by the fact that fragments of its human homologue FIP2 were isolated with the yeast two-hybrid system by two different groups (Faber et al., 1998; Li et al., 1998). In serial deletion mutant analysis, one of the leucine-zipper domains, the more C-terminal one, proved to be required in the interaction of FIP2 with a virus protein that functions in the apoptosis pathway (Li et al., 1998). FIP3 was also

shown to interact with E3 14.7K protein and Fas receptor with its C-terminus and middle part, respectively (Ye et al., 2000).

Interestingly, an ERM domain was located in 9C5 during a search for conserved domains (http://www.ncbi.nlm.nih.gov/Structure/cdd). The middle 150 amino acids (226-282) of 9C5 align with this conserved domain (Figure 4, 6). ERM is short for the Ezrin/radixin/moesin family of proteins (Yonemura et al., 1998). All three of these proteins are located in the microvillar peripheral area of polarized cells and are involved in the connection of major cytoskeletal structures to the plasma membrane (Bergson et al., 1993; Lankes and Furthmayr, 1991; Wilgenbus et al., 1993). They are shown to bind to the cytoplasmic domains of CD44 (Tsukita et al, 1994) and other integral membrane proteins (Yonemura et al., 1998; Yonemura et al., 1993) through their ERM domain. The binding is required for many cell morphogenesis events, such as microvillus formation during polarization (Yonemura et al., 1999).

Under cell-cell contacting surfaces, some molecules, for example, α-catenin, have been identified to link E-cadherin to actin cytoskeleton, and the connection is required in polarization process (Rimm et al., 1995). ERM group proteins are suggested to play the same role under the noncontacting (apical) region membrane (Bergson et al., 1993; Lankes and Furthmayr, 1991; Wilgenbus et al., 1993). It is not clear if 9C5 functions in a similar way as other ERM proteins. It could bind cytoskeletal proteins or other mediators to help connect the apical membrane with the subcanalicular cytoskeletal network in hepatocytes, although there isn't an actinin-binding site, as defined by PsortII sequence analysis (http://psort.nibb.ac.jp, (Nakai and Horton, 1999)). Further study of the interaction between 9C5 and cytoskeleton components will help us understand if 9C5 is

involved in the formation of subcanalicular cytoskeletal network and has functions similar to the other ERM family proteins.

Such a submembranous protein scaffold normally also serves to integrate signaling of polarization and interact with downstream signaling pathways (Yeaman et al., 1999). 9C5 could also participate in relaying signals to or from the apical compartment. Kinases and phosphatases have been implicated in a signaling pathway that is required in the formation of the submembrane cytoskeleton network and eventually the polarization of cells (Brady-Kalnay et al., 1995; Clark and Brugge, 1995; Lewis et al., 1994; Tsukita et al., 1991). The 9C5 protein contains many potential phosphorylation sites. Some other signaling molecules, such as Rho family GTPases, are heavily involved in regulating cell adhesion, polarity and actin organization (Kaibuchi et al., 1999; Schwartz and Shattil, 2000; Tsukita and Yonemura, 1999). They are shown to function both upstream (Hirao et al., 1996) and downstream (Takahashi et al., 1997) of ERM family proteins. It will be important to determine if 9C5 interacts with these molecules and if 9C5 is associated with the signal transduction pathway during polarization of hepatocyes.

In contrast to its localization in polarized cells, 9C5 shows a punctate expression pattern in unpolarized cells. The punctate localization pattern suggests 9C5 may travel with small vesicles in the cytoplasm. In hepatocytes, the apical proteins are transported to the basolateral surface first and then they are redirected to the apical surface through transcytosis (Bartles et al., 1987). It is believed that the sorting of proteins happens in the TGN by inclusion into different transport vesicles that carry proteins to their correct destinations (Griffiths and Simons, 1986). Different signals are used for apical proteins

and basolateral proteins. The interaction between the signal portion of membrane proteins and the cytosolic proteins decides the selection of different transcytotic vesicles. Before polarization, 9C5 appears to be punctately located in cytoplasm and possibly associate with small vesicles. Upon polarization, 9C5 quickly shifts to the subcanalicular area or canalicular domain (Gallin and Sanders, 1992). Most likely, 9C5 is one of the passengers that are carried to the subapical area by the protein sorting system, where it becomes part of the subcanalicular protein scaffold complex. However, it is also possible that 9C5 may also act as part of the sorting and selection machinery to direct the apical proteins to their destined localizations in hepatocytes. With its potential membrane protein binding activity (ERM domain), 9C5 could potentially help lead apical proteins to their destinations.

All the five monoclonal antibodies stain various epithelial tissues other than liver and four of them are only expressed along the apical surface in all the tested tissues (Gallin and Sanders, 1992). 9C5 is not restricted to the apical surface in other epithelial tissues. It is present throughout the cytoplasm in a grainy pattern in kidney and other non-liver epithelial cells (Gallin and Sanders, 1992), which correlates with our immunofluorescence results in transfected MDCK, a kidney-derived cell lines (Figure 10, 11). Different mechanisms are involved in the establishment and maintenance of the cell polarity in hepatocytes and other epithelial cells. In MDCK cells, for example, there is a direct route for apical targeting (Bartles et al., 1987). The unique pericanalicular filaments, which are part of the complicated subapical organization, might also contribute to the different properties of hepatocytes. The different behaviors of 9C5 in different cell types are possibly related to the different subapical component organization or different

protein sorting schema. Both 9C5 itself and tissue-specific factors that interact with 9C5 can contribute to the tissue-specific localization of 9C5. Further study of 9C5 might provide insight into why and how the unique apical targeting and subapical organization is utilized in hepatocytes.

The 9C5 human homologue FIP2 was isolated in yeast two-hybrid assay, searching for interacting cellular proteins of the Adenovirus E3 14.7-K protein that can block apoptosis of host cells. Another protein, FIP3, was also picked up in the same screening process (Li et al., 1999b; Li et al., 1998). FIP3 also contains leucine zippers and zinc finger domain and shares 26% identity with FIP2 (Li et al., 1999b). FIP3 later was demonstrated to bind RIP and NIK and inhibit the activation of NF-κB induced by TNF-α (Ye et al., 2000). Its mouse homologue was isolated later and shown to bind IKKγ (Yamaoka et al., 1998). It is recently demonstrated that FIP3 might serve as a scaffolding protein to form the multiple-component IKK complex (Ye et al., 2000). Different functions of FIP3, which includes IKKα and IKKβ binding, RIP-binding, NF-κB inhibition, etc, have been mapped to different domains of FIP3 including the three leucine zippers and the zinc finger (Ye et al., 2000). FIP3 seems to serve as a scaffold protein to organize a multimeric complex (Ye et al., 2000). Taken together, FIP2, 9C5 and FIP3 all contain similar motifs at similar positions and possibly share a conserved domain structure (Figure 17).

During the differentiation of hepatocytes, different sets of genes are expressed at different stages and NF-κB is thought to play a very active role in the regulation of gene expression and apoptosis in hepatocyes (Duncan, 2000). FIP3 has already been demonstrated to be involved in the NF-κB pathway (Heyninck et al., 1999; Ye et al.,

2000). FIP2 reverses the antiapoptotic effect of the adenovirus protein and it could possibly do so by repressing the NF-κB pathway, which is antagonistic route of apoptosis. If this is the case, these proteins seem to share a similar function as well. We propose that they belong to a new class of protein family with common motifs and possibly similar 3-D structures. These proteins are mostly related to NF-κB activation and are important mediators in this pathway that is essential to many physiological activities, such as apoptosis and gene expression. The possible roles of 9C5/FIP2 in cell differentiation/polarization and apoptosis do hint it might be involved in a multiple-role pathway, such as the NF-κB pathway. Further study of the proteins with the same domains and identification of 3-D structures will tell us more about these proteins.

Finally, the 9C5 human homologue FIP2, as well as data from FIP3, allows us to tentatively put 9C5 into the apoptotic pathway. It is possible that 9C5 is part of the apoptosis regulating pathway in hepatocytes and it may also be linked to the process of polarization of hepatocytes and maintenance of bile canaliculi. It has been suggested that apoptosis plays an important role in maintaining hepatocyte turnover in the adult liver. Targeted mutation of fas, which mediates Fas ligand-induced apoptosis, results in liver hyperplasia caused by an accumulation of senescent hepatocytes (Adachi et al., 1995). During the constant remodeling of the bile canaliculus network, the turnover and life-death balance of the cells on the canalicular surfaces are far more critical than other liver parenchymal cells, because the minimal requirement for a functional bile canaliculi is a complete network without any leaks, which are presumably due to "unprogrammed" cell death. Apoptosis certainly would be the choice these cells use to program and regulate their destiny and balance the total number of the bile canalicular cells. The anti-TNF-α-

induced apoptotic effect of NF-κB in the differentiating hepatocytes has been demonstrated to be important in the development of liver (Rosenfeld et al., 2000). The human protein FIP2 was TNF-α inducible (Li et al., 1998). Therefore, it is possible that 9C5/FIP2 may be able to sense the information in abnormally differentiated hepatocytes and initiate apoptosis. They may not directly be part of the apoptosis pathway, rather it could affect the apoptotic route by regulating NF-κB activation, as other proteins with the same domains do. Considering the apoptosis inducing effect of overexpressed FIP3 (Ye et al., 2000), it also possible that FIP2/9C5 is involved in the apoptosis pathway directly, although it hasn't been shown to induce cell death by itself (Li et al., 1999b; Li et al., 1998).

The C-terminus of FIP2 was shown to interact with the N-terminus of Huntingtin, the causative protein in Huntinton's disease. Huntingtin has been shown to be expressed in the liver (Hoogeveen et al., 1993). The N-terminus of Huntingtin is where the polymorphic CAG trinucleotide repeats are. Apoptosis has been proposed to be involved in the pathogenesis of Huntington's disease. Increased apoptosis was demonstrated in mice nullizygous for the Huntingtin's mouse homologue (Zeitlin et al., 1995). Apopain, the human caspase 3, was found to specifically cleave the Huntintin protein and the rate of cleavage increases with the length of the N-terminal polyglutamine tract (Goldberg et al., 1996). Therefore, Huntingtin was thought to counterbalance the operation of the apoptotic pathway. The association of FIP2/9C5 with Huntingtin might link the NF-κB pathway and Huntingtin together, to indirectly interfere with the apoptotic process.

In summary, we constructed VSV-G tagged 9C5 expression plasmids. The immunofluorescence results with these constructs show that the expression of VSV-9C5

and its localization in different cell lines are in accordance with the endogenous 9C5 antigen. Both 3' and 5' VSV-9C5 gave similar data, so the VSV-G tag doesn't affect its cellular expression and localization. The VSV-9C5 constructs possibly carry the same physiological activities as the endogenous 9C5 protein. With these constructs, one can look further into the structure and function of 9C5 protein, and its roles in the development of the bile canaliculus network.

From the sequence of 9C5 and its localization in polarized hepatocytes, 9C5 is likely to be part of the subcanalicular complex and act as a scaffold protein to recruit signaling molecules during polarization of hepatocytes. Searching for and characterizing its interacting partners will help one to clarify this issue, and a perturbation approach will allow one to know more about the functions of 9C5. The data from human protein FIP2 and FIP3 suggest that 9C5 might also be involved in the NF-κB pathway. It will be important to see if 9C5 can affect the activation of NF-κB, which will provide one with more information about 9C5 and the regulation of NF-κB signaling pathway.

Figure 1. Polarization of epithelial cells. Epithelial polarization is thought to be initiated by cell adhesions. A submembranous complex is then assembled under the cell-contacting surface and responsible for propagating signals to inside of cells. Upon receiving the signal, protein sorting machinery will start delivering proteins to different membrane domains according to their destinations.

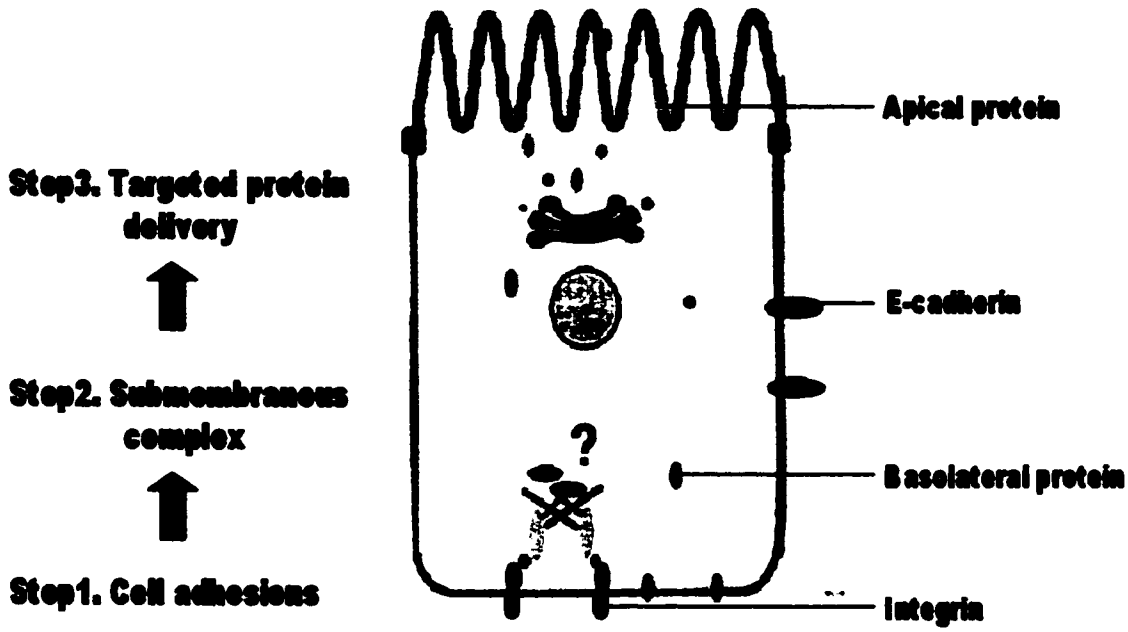


Figure 1. Polarization of epithelial cells.

Figure 2. Map of sequence data that 9C5 was assembled from. Automated sequencing has been done with ten internal primers with clone 71 as template. All isolated clones and one subclone were sequenced from both ends using T3 and T7 sequencing primers. The probe used in Northern analysis is labelled in blue. Arrows stand for valid sequence data obtained from each sequencing reaction.

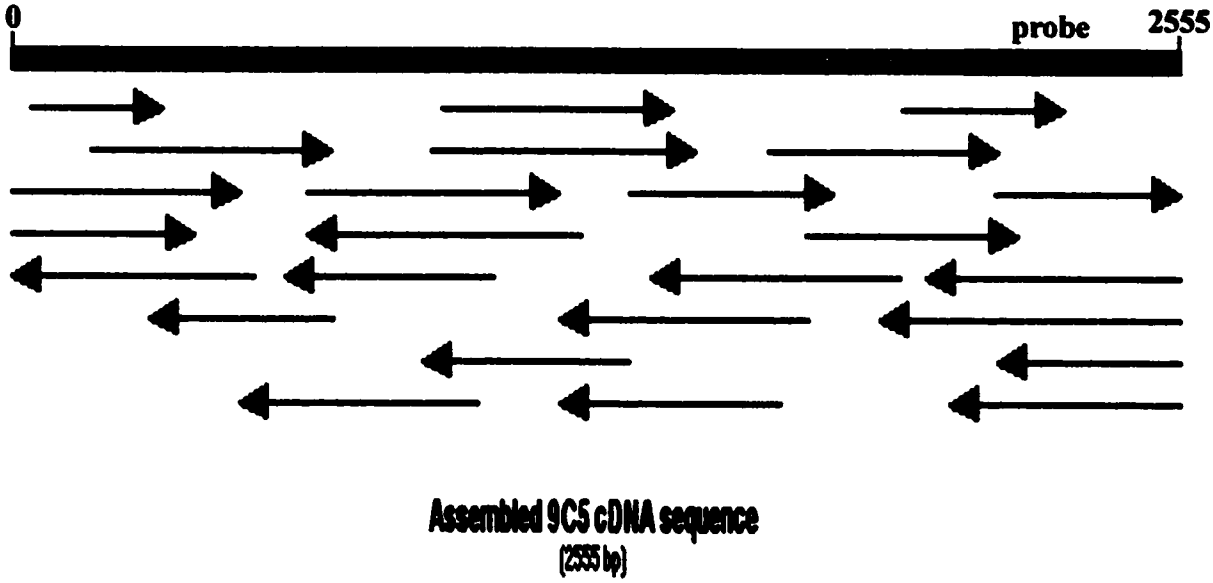


Figure 2. Sequence data that 9C5 was assembled from.

Figure 3. Blotting of phage clone 62 with individual monoclonal antibodies. A single membrane lift from plated purified phage 62 was cut into six fragments and each was blotted individually with one of the canaliculus monoclonal antibodies, or with no primary antibody. Only antibody 9C5 reacts with clone 62. The same result was obtained with the other isolated phage clones as well.

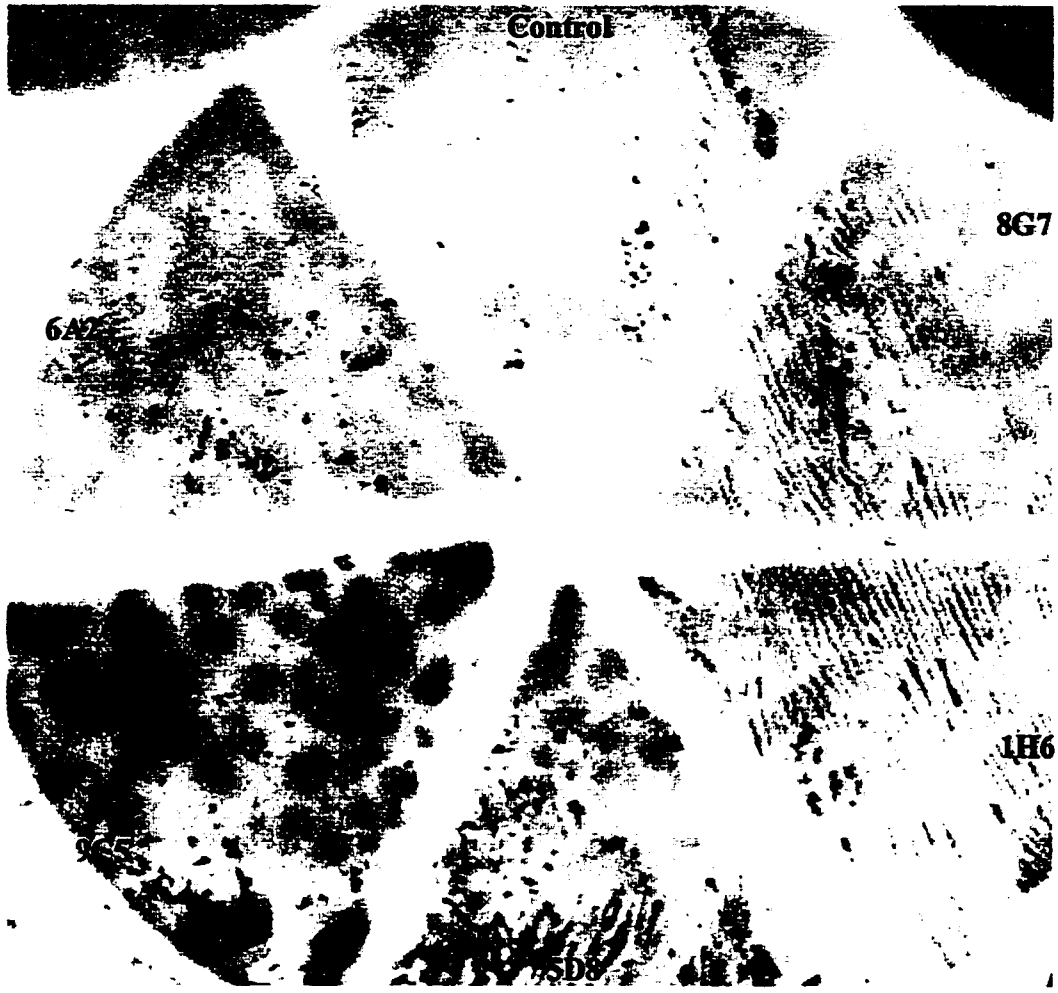


Figure 3. Antigenic specificity of 9C5 clone.

Figure 4. Comparison of isolated six individual cDNA clones and the assembled 9C5 sequence. The assembled sequence is 2555 bp long. The start and stop codons of the predicted 9C5 coding region are labelled. The ERM domain is highlighted in dark blue. The divergent part of Clone b is labelled in light blue. The spans of all the clones are indicated by their start and stop positions.

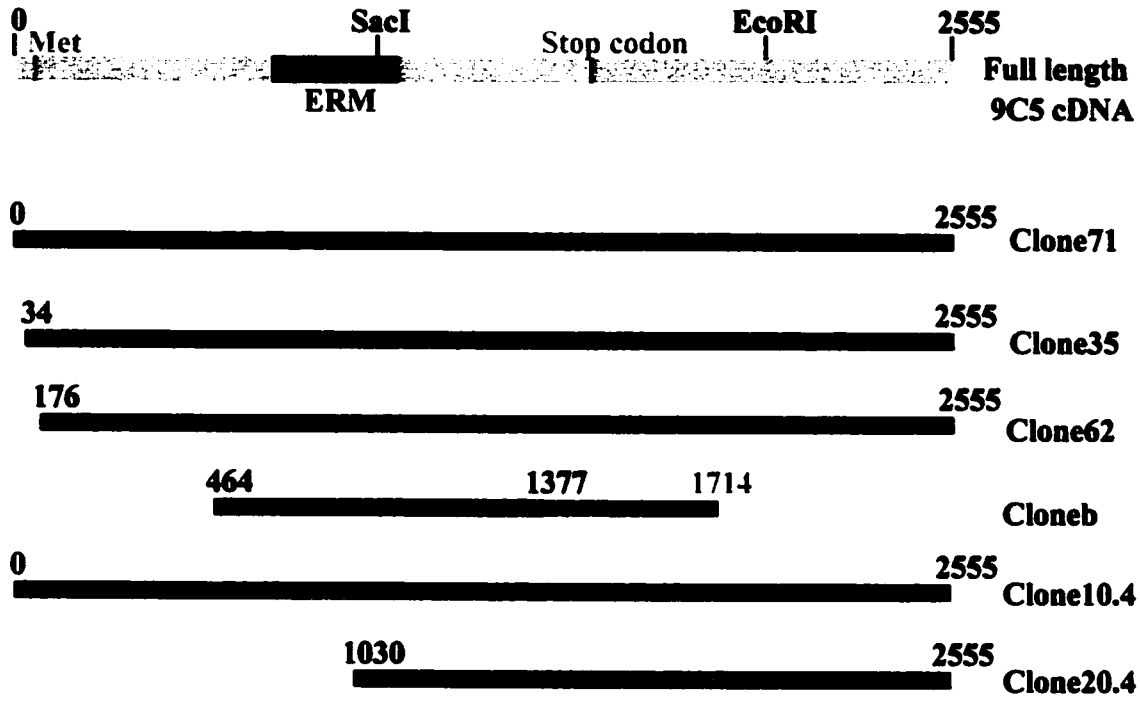


Figure 4. Isolated cDNA clones by canalicular marker Ab.

Figure 5. Result of BLAST searching using predicted 9C5 protein sequence. A human protein FIP2 was hit with a score of 557 and an E-value of e-157. The huntingtin interacting protein is actually the C-terminal end of FIP2. FIP3 also shares high similarity with 9C5 and it is a component of the IKK complex. The red box includes the group of proteins with relatively higher scores. Those hits after them are proteins such as myosin heavy chain, which has a coiled-coil structure. The scores of these coiled-coil proteins are most likely due to the predicted long range coiled-coil structure of 9C5.

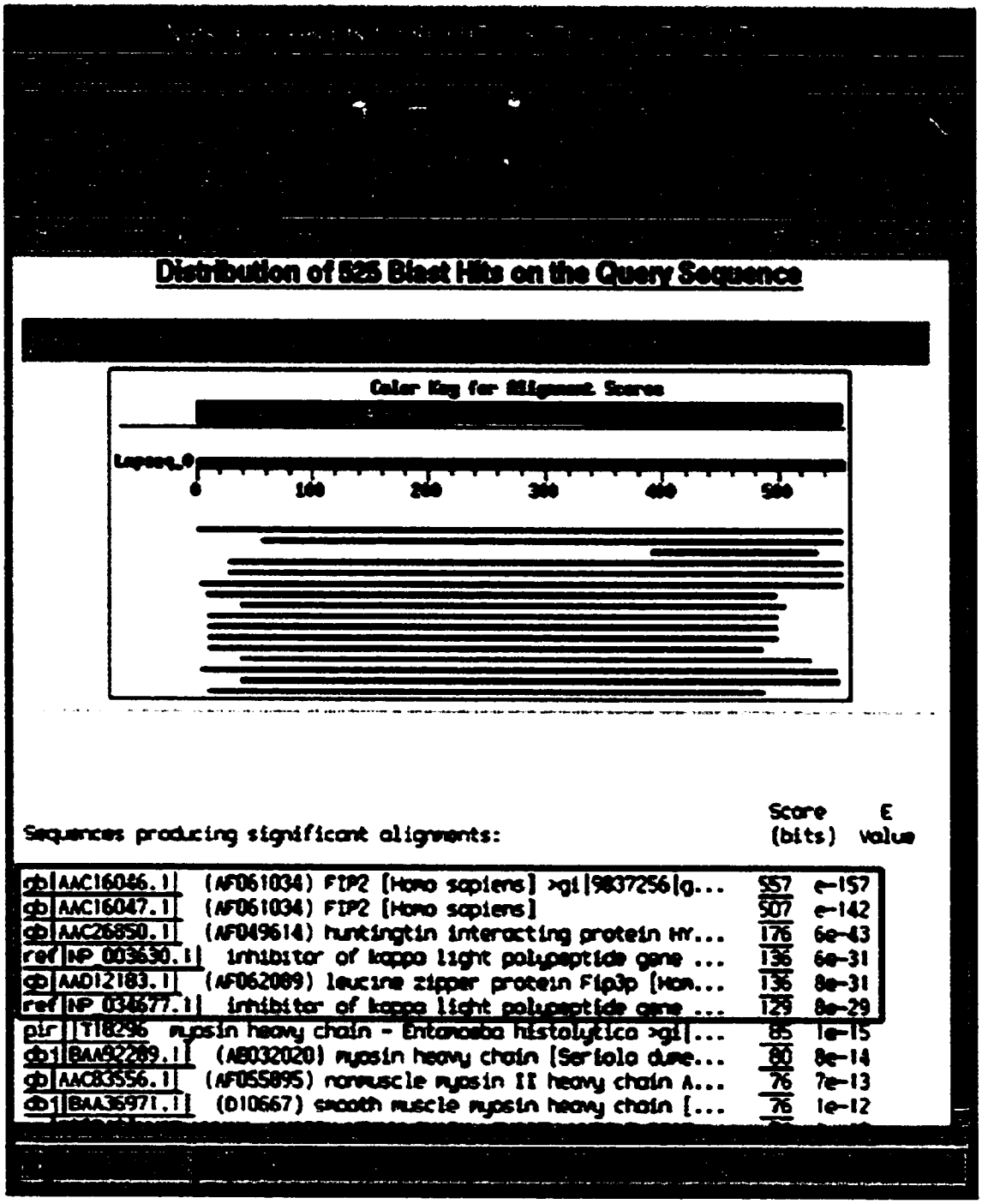


Figure 5. BLAST search of 9C5

Figure 6. Alignment of 9C5 with its human homologue FIP2. The alignment was done by GeneDoc (Nicholas et al., 1997). 9C5 shares 52% identity with FIP2 protein. Identical amino acids are labelled in dark blue. Three leucine zippers are labelled in red. The zinc finger is also labelled at the very C-terminal end. The ERM domain is labelled in the middle part of the sequence.

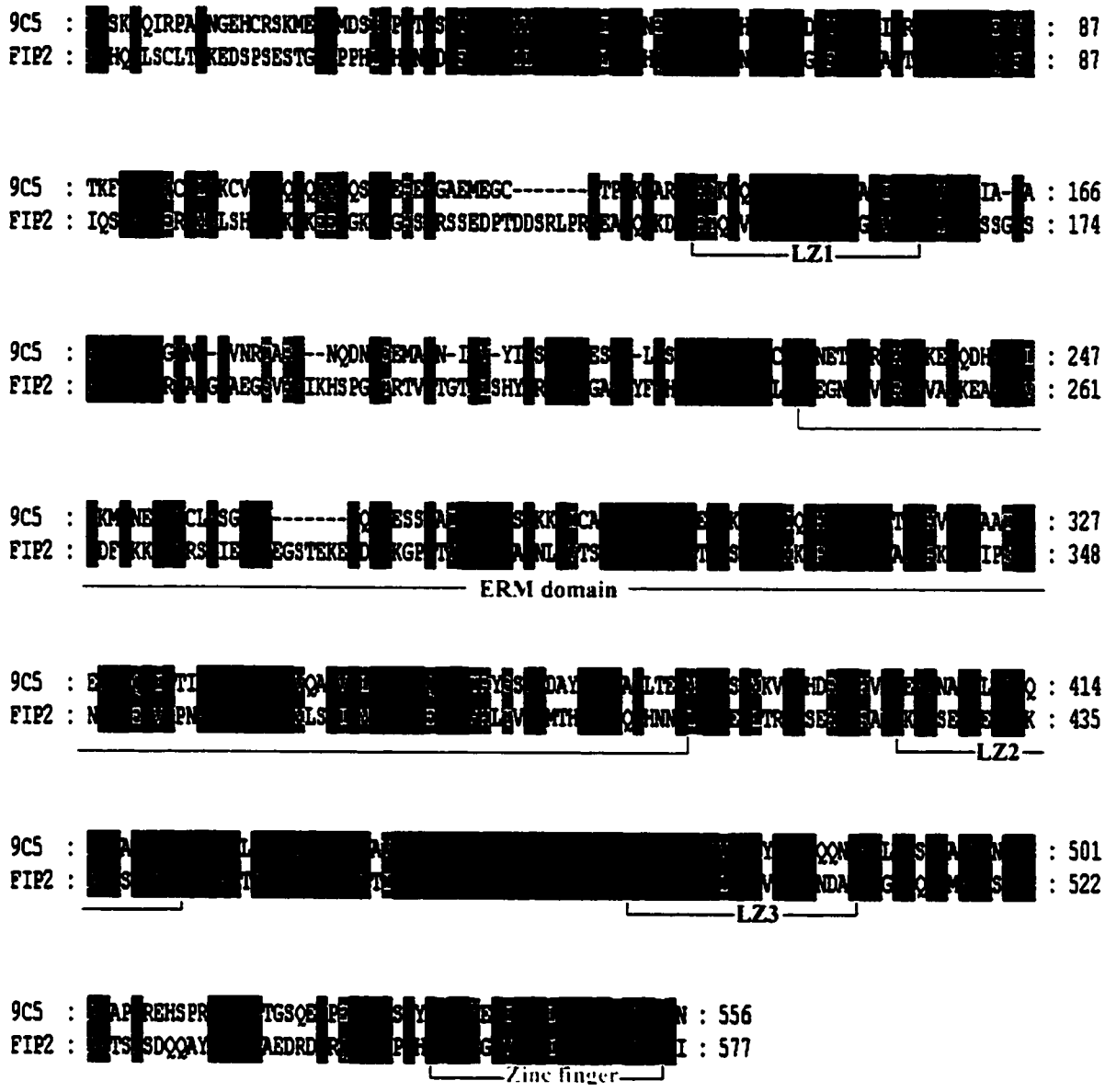


Figure 6. Alignment of 9C5 with FIP2.

Figure 7. Northern blotting of total RNA from embryonic chicken organs with a 9C5 cDNA probe. Hybridization was done with 10 μg total RNA from different chicken embryo organs. P^{32} was used to label the probe, which is a 600 bp fragment from the 3' end of isolated clone 35 (Figure 2). The blot was visualized using Phosphoimager (Molecular Dynamics). The expression of 9C5 is detected in chicken liver, heart, muscle, brain and kidney. There is no detectable signal in lung. The only detectable band is located at approximately 2600-2800 bp range.

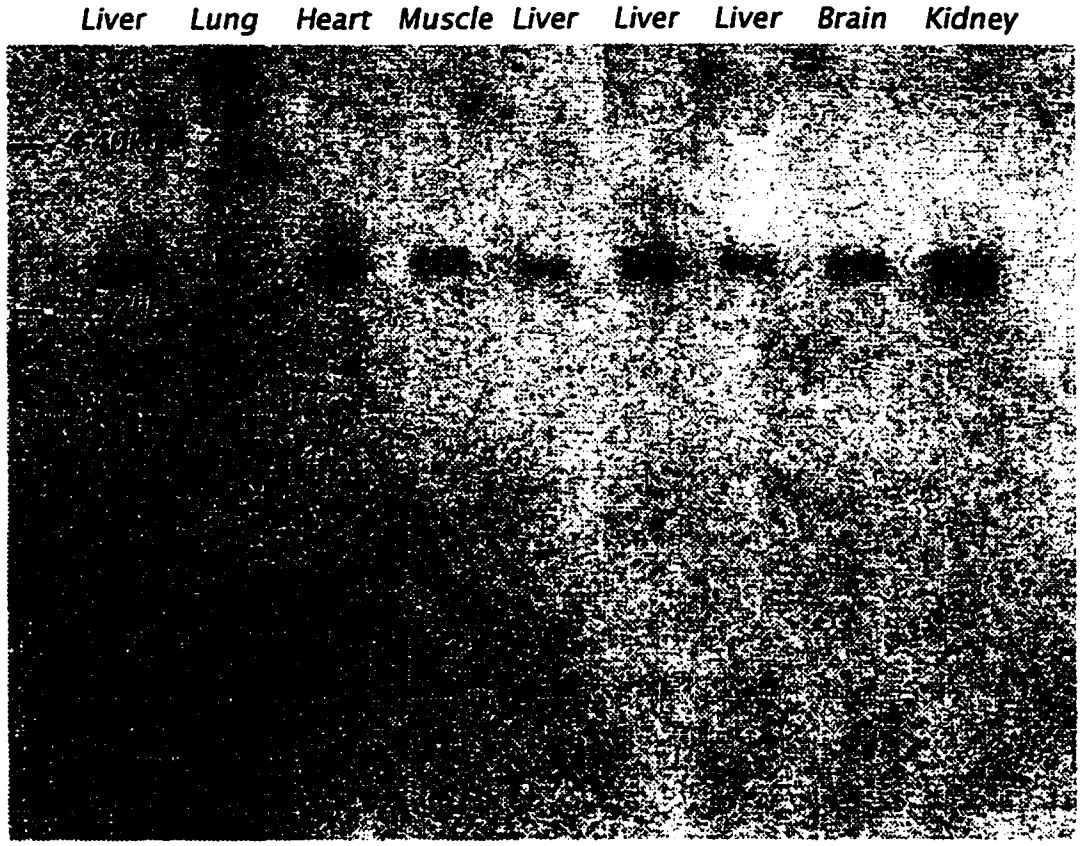


Figure 7. Northern analysis of 9C5.

Figure 8. Maps of epitope-tagged 9C5 expression plasmids. An eleven amino acid tag VSV-G was added onto either the 5' or 3' end of the 9C5 coding region with the help of PCR. Both constructs are in expression vector pBK-CMV. Blue arrows indicate the orientation of 9C5 transcription. VSV-G tags are labelled in green. The NheI and EcoRI sites were used to ligate the insert in the 3' VSV-9C5 construction and BamHI and EcoRI sites were used in the 5' VSV-9C5 construction.

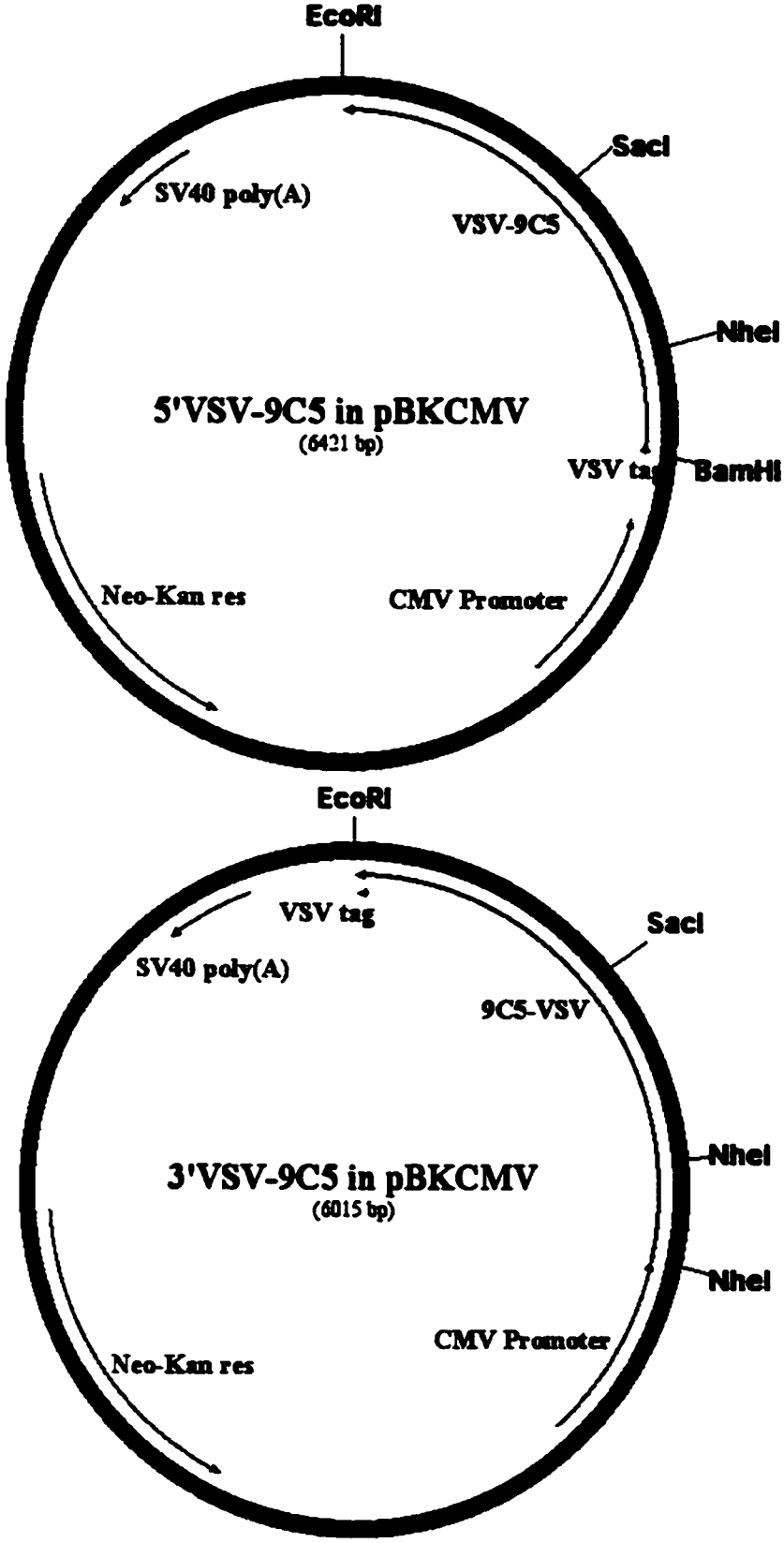


Figure 8. Plasmid construction.

Figure 9. Fluorescent staining of transfected and untransfected Cos7 cells.

A) Pmaori plasmid was transfected into Cos7 cells with PEI and detected by anti β-galatosidase Ab and FITC conjugated secondary Ab. Pmaori contains a lacZ gene. All stainings are located in the nuclei.

B) 5'VSV-9C5 was transfected into Cos7 cells with PEI and detected by anti VSV-G Ab and FITC conjugated secondary Ab. It shows punctate cytoplasmic staining only. The nucleus is marked with an asterisk.

C) 3'VSV-9C5 was transfected into Cos7 cells with PEI and treated with FITC conjugated secondary Ab only (no anti VSV-G Ab). No signal is detected.

D) Untransfected Cos7 cells was treated with anti VSV-G Ab and FITC conjugated secondary Ab. No signal is detected.

E) Untransfected Cos7 cells was treated with 9C5 Ab and FITC conjugated secondary Ab. No signal is detected.

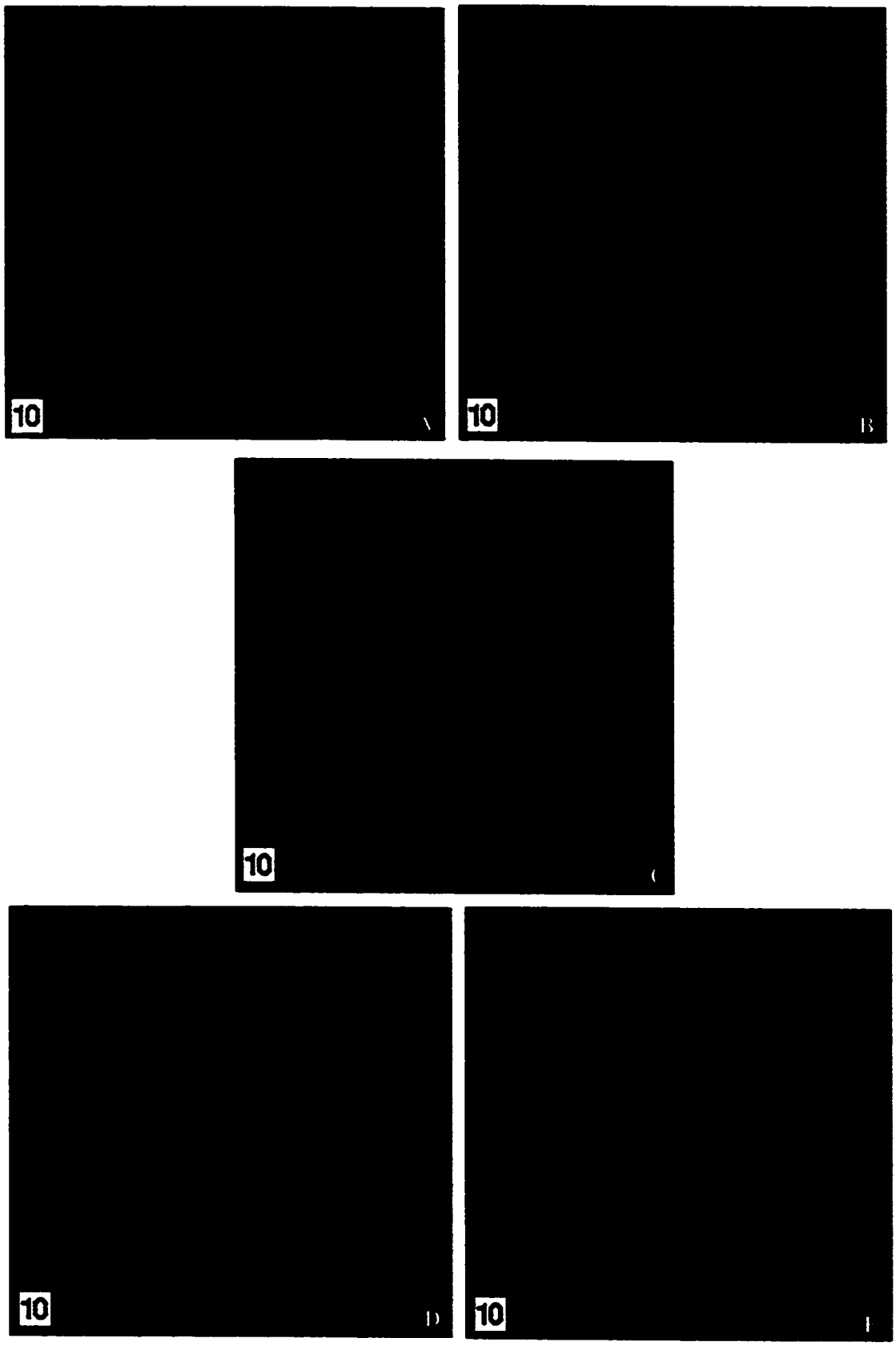
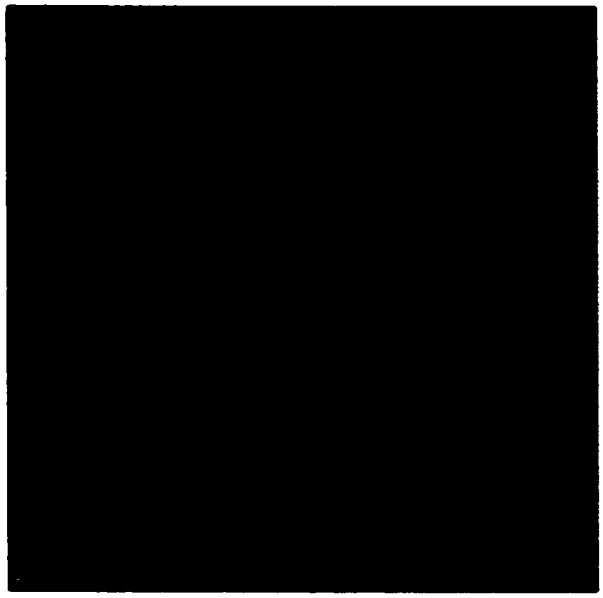
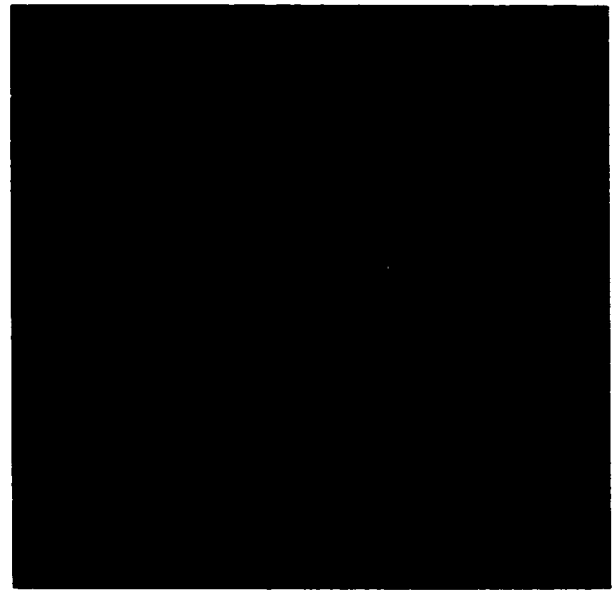


Figure 9. Transfection in Cos 7 cells.

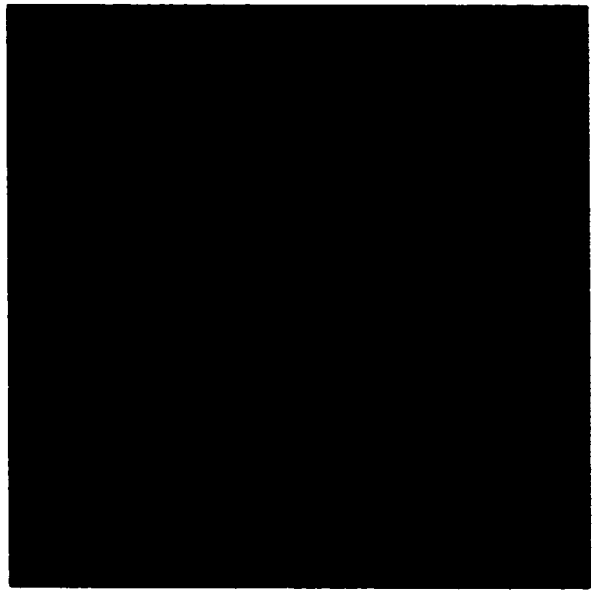
Figure 10. Fluorescent staining of transfected Cos7 cells with VSV-G Ab and 9C5 Ab. Both 3'VSV-9C5 and 5'VSV-9C5 were transfected into Cos7 cells with PEI. FITC conjugated secondary Ab was used. Both constructs show punctate cytoplasmic staining. The nuclei are labelled with asterisks. The expressed proteins can be detected by both anti VSV-G Ab and 9C5 Ab.



5'VSV-9C5 in Cos7 cells
VSV Ab



3'VSV-9C5 Cos7 cells
VSV Ab

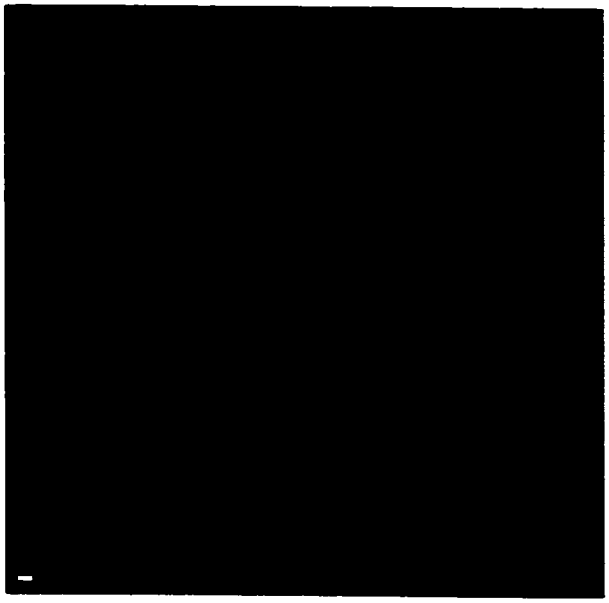


5'VSV-9C5 in Cos7 cells
9C5 Ab

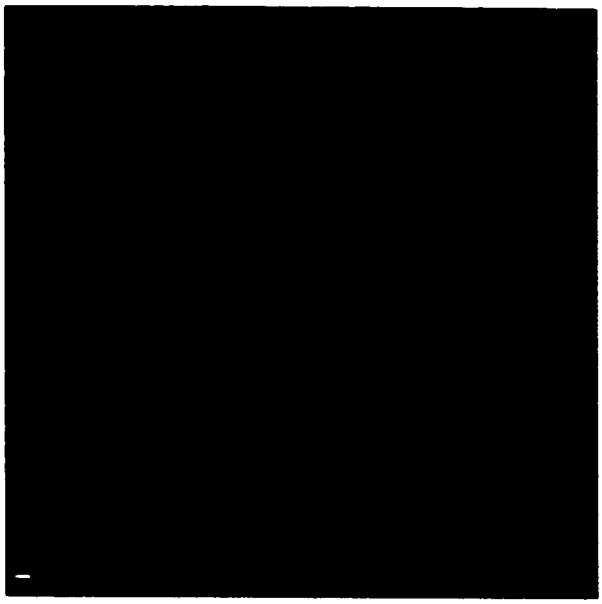
3'VSV-9C5 in Cos7 cells
9C5 Ab

Figure 10. Transfection in Cos 7 cells.

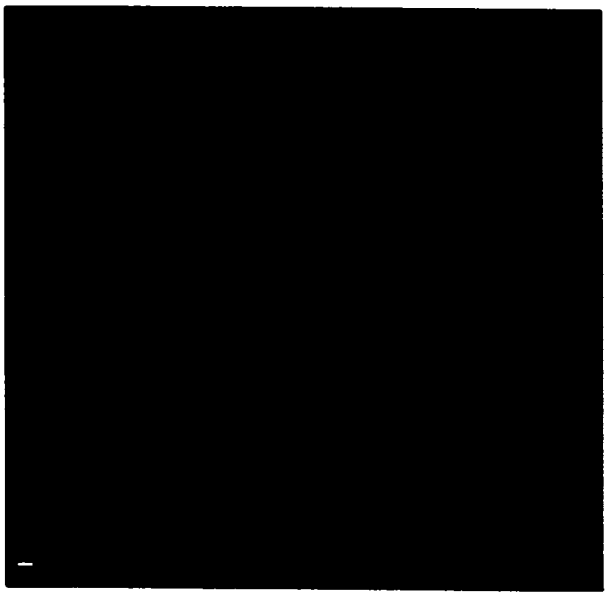
Figure 11. Fluorescent staining of transfected and untransfected MDCK cells. 3'VSV-9C5 was transfected into MDCK cells with PerFect transfection kit (Invitrogen) and detected by VSV Ab and FITC conjugated secondary Ab. It shows cytoplasmic staining and the nucleus is labelled with an asterisk. There is no detectable signal in transfected MDCK cells treated with secondary Ab only. No signal is detected in untransfected MDCK cells with either anti VSV-G Ab or 9C5 Ab.



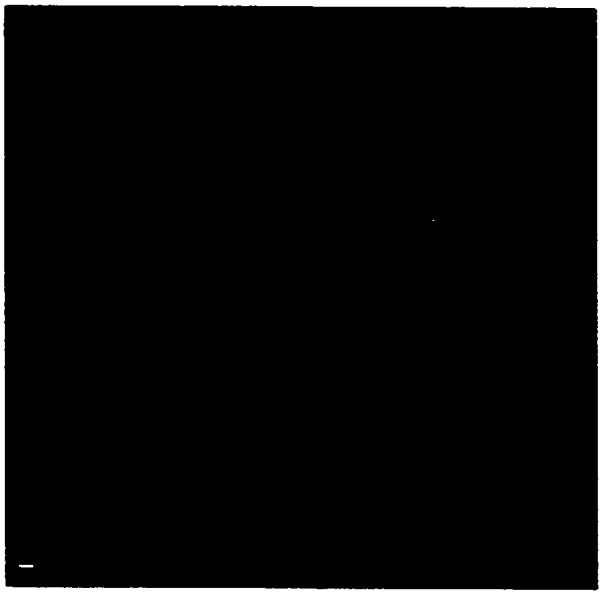
3'VSV-9C5 in MDCK cells
VSV Ab



3'VSV-9C5 in MDCK cells
secondary Ab only



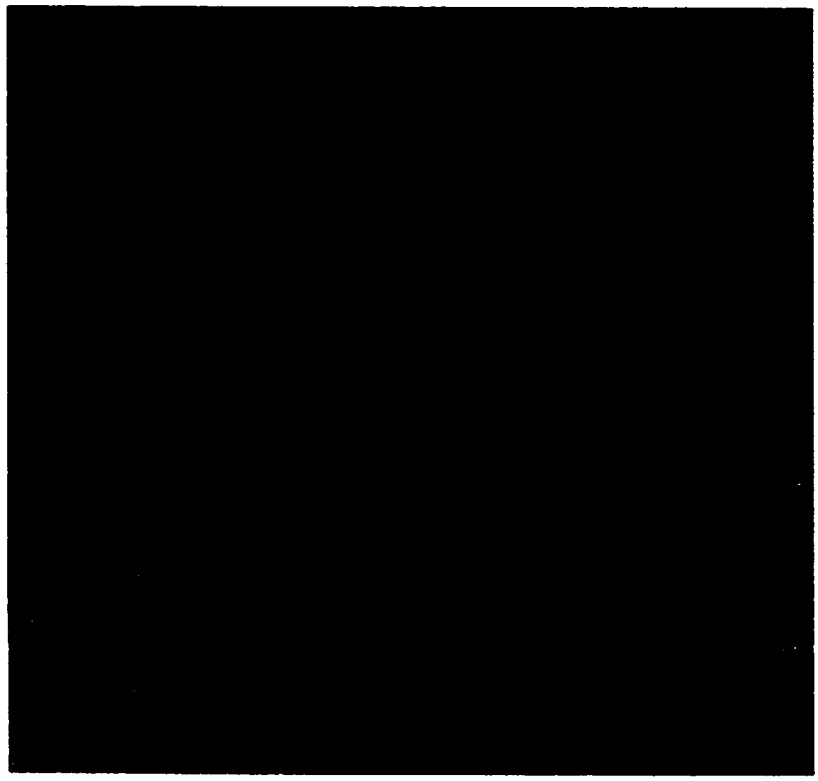
Untransfected MDCK cells
VSV Ab



Untransfected MDCK cells
9C5 Ab

Figure 11. Transfection in MDCK cells.

Figure 12. Vertical distribution of fluorescent signal of a VSV-9C5 transfected MDCK cell. The transfected MDCK cell was scanned along the vertical axis (Z-axis) of the cell to locate the signal using a laser confocal microscope. Fluorescence signals are detected along the vertical axis. Signals in both Y-Z and X-Z axes spread along the vertical axis. It indicates that signals are not limited to the top of MDCK cells, where the apical surface is. The quantitation analysis also shows high signals at middle part along the vertical axis of the cell. The scanning was done over 7μm distance, which is roughly the thickness of a single cell.



3'VSV-9C5 in MDCK cells

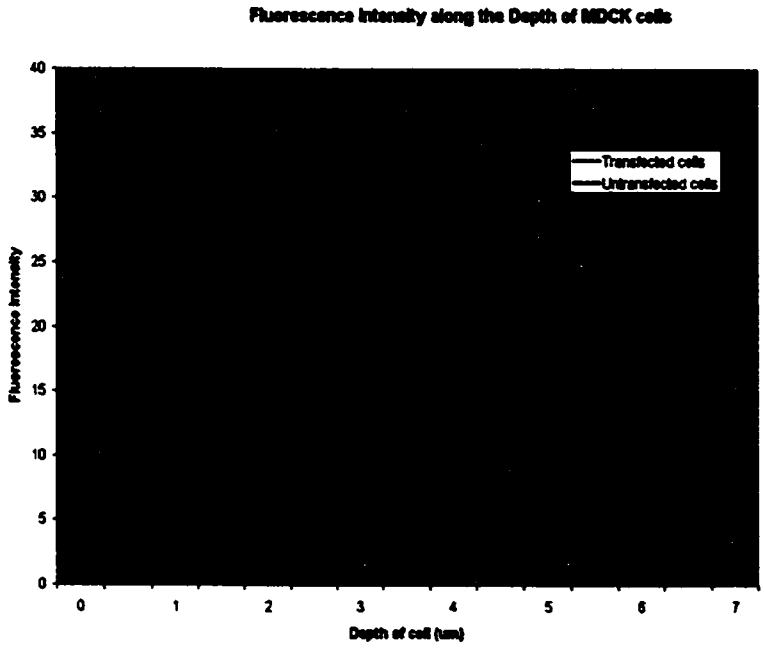
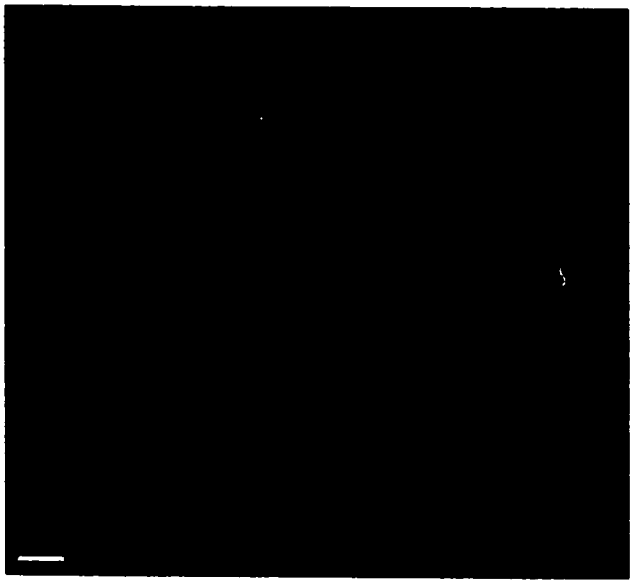
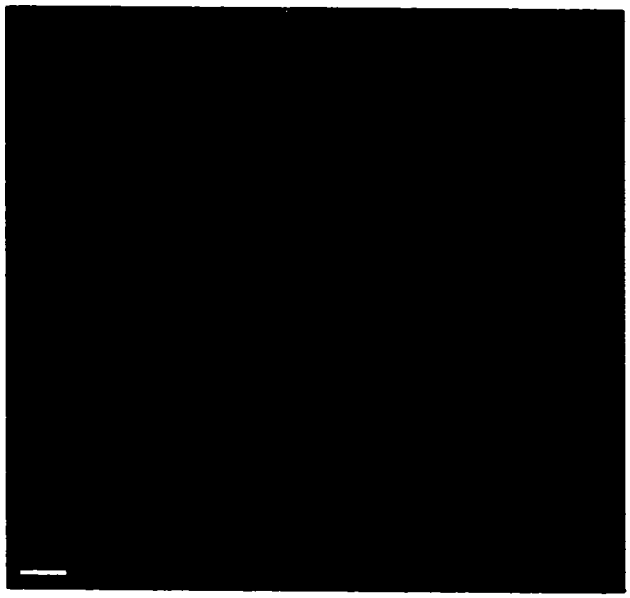


Figure 12. 3'VSV-9C5 in MDCK cells.

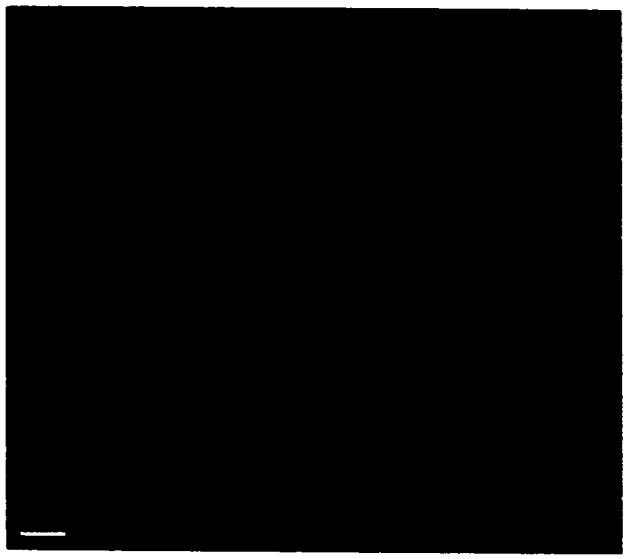
Figure 13. Fluorescent staining of untransfected liver cells. Untransfected 8-day chicken liver cells were detected with 9C5 Ab and anti VSV-G Ab, with FITC and Rhodamine conjugated secondary Abs. 9C5 Ab staining shows a network pattern. No signal is detected with anti VSV-G Ab in untransfected liver cells.



Untransfected liver cells
9C5 Ab



Untransfected liver cells
VSV Ab

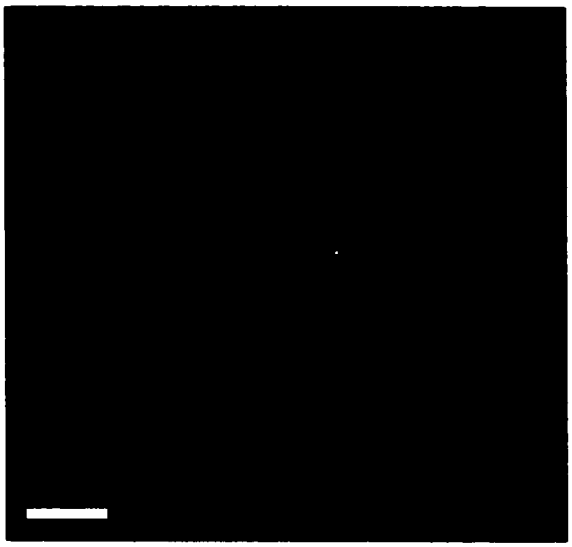


Untransfected liver cells
9C5 Ab and VSV Ab

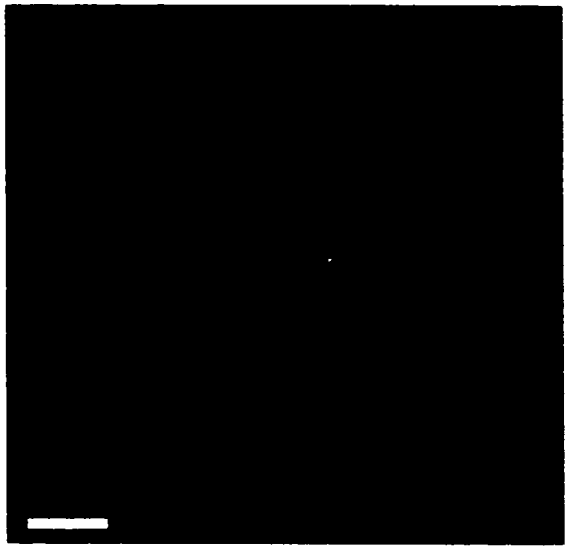
Figure 13. Untransfected liver cells.

Figure 14. Fluorescent staining of 3'VSV-9C5 transfected liver cells. 3'VSV-9C5 was transfected into 8-day chicken liver cells by biolistic transfection and detected with 9C5 Ab and anti VSV-G Ab, with FITC and Rhodamine conjugated secondary Abs. 9C5 Ab stains the bile canaliculus network (green) while anti VSV-G Ab stains cells that have been tranfected with 3'VSV-9C5 (red). The anti VSV-G staining is colocalized with 9C5 staining, as indicated by arrows.

3'VSV-9C5 in liver cells
9C5 Ab



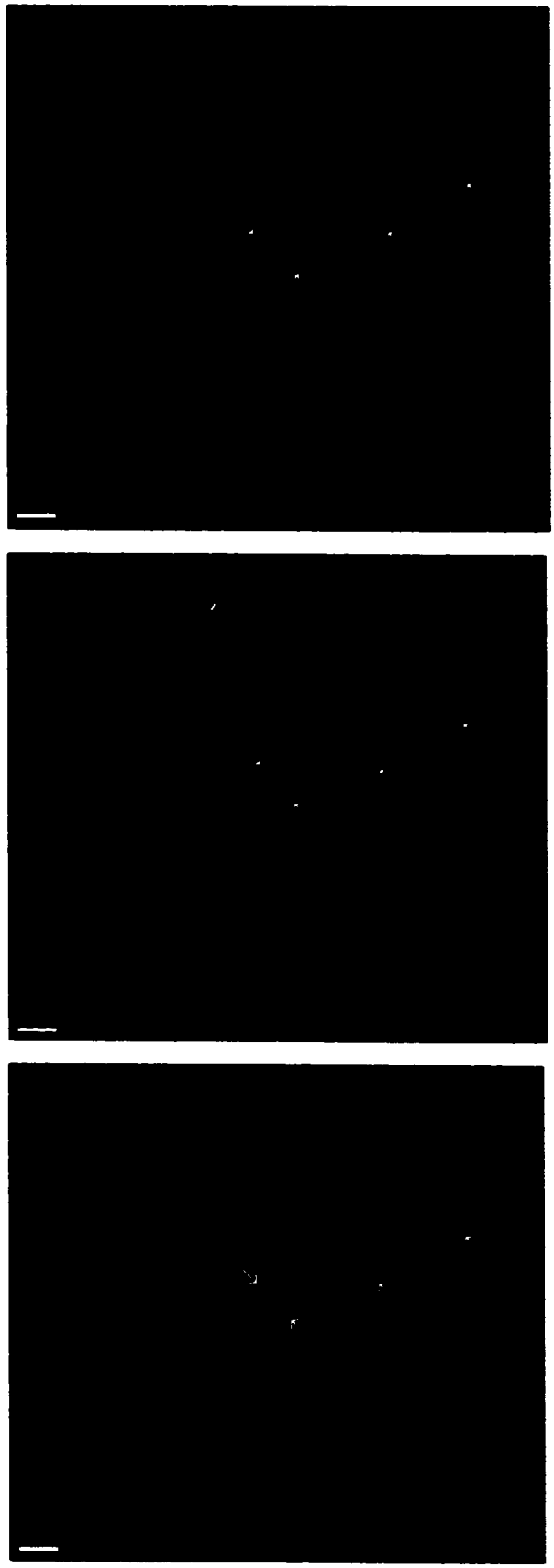
3'VSV-9C5 in liver cells
VSV Ab



3'VSV-9C5 in liver cells
9C5 Ab and VSV Ab

Figure 14. 3'VSV-9C5 in liver cells.

Figure 15. Fluorescent staining of 5'VSV-9C5 transfected liver cells. 5'VSV-9C5 was transfected into 8-day chicken liver cells with biolistic transfection and detected with 9C5 Ab and anti VSV-G Ab, with FITC and Rhodamine conjugated secondary Abs. 9C5 Ab stains the bile canaliculus network (green) while anti VSV-G Ab stains cells that have been tranfected with 5'VSV-9C5 (red). The anti VSV-G staining is colocalized with 9C5 staining, as indicated by arrows.



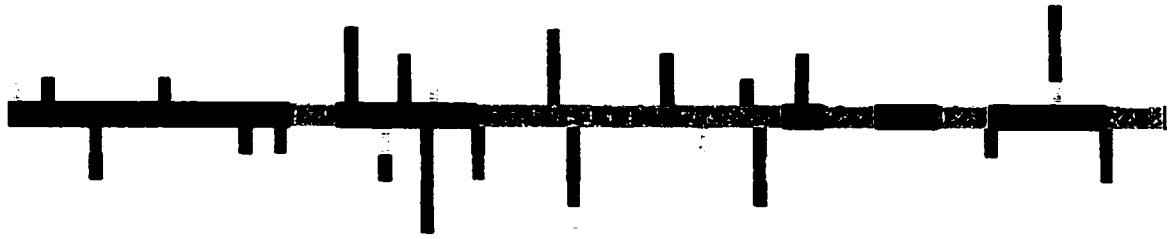
5'VSV-9C5 in liver cells
9C5 Ab

5'VSV-9C5 in liver cells
VSV Ab

5'VSV-9C5 in liver cells
9C5 Ab and VSV Ab

Figure 15. 5'VSV-9C5 in liver cells.

Figure 16. Potential phosphorylation sites of 9C5. The phosphorylation sites were predicted by Peptool (Biotools). The three leucine zippers (LZ) and the zinc finger (ZF) are labelled. The ERM domain is also labelled. Different phosphorylation sites are marked with different colors, as listed in the figure. Although no phophorylation site was identified within the conserved motifs, there are several sites predicted to be around the borders of LZ3 and ZF domains.



Protein kinase C

Casein kinase I

Glycogen synthase kinase 3

Casein kinase II

S6 kinase II

Myosin 1 heavy chain kinase

Multifunctional calmodulin dependent kinase

Proline dependent kinase

Figure 16. Phosphorylation sites of 9C5.

Figure 17. Structural domains of 9C5, FIP2 and FIP3. The leucine zippers are labelled in red and the zinc finger domain is labelled in green. These three proteins share structural domains at the same relative positions.

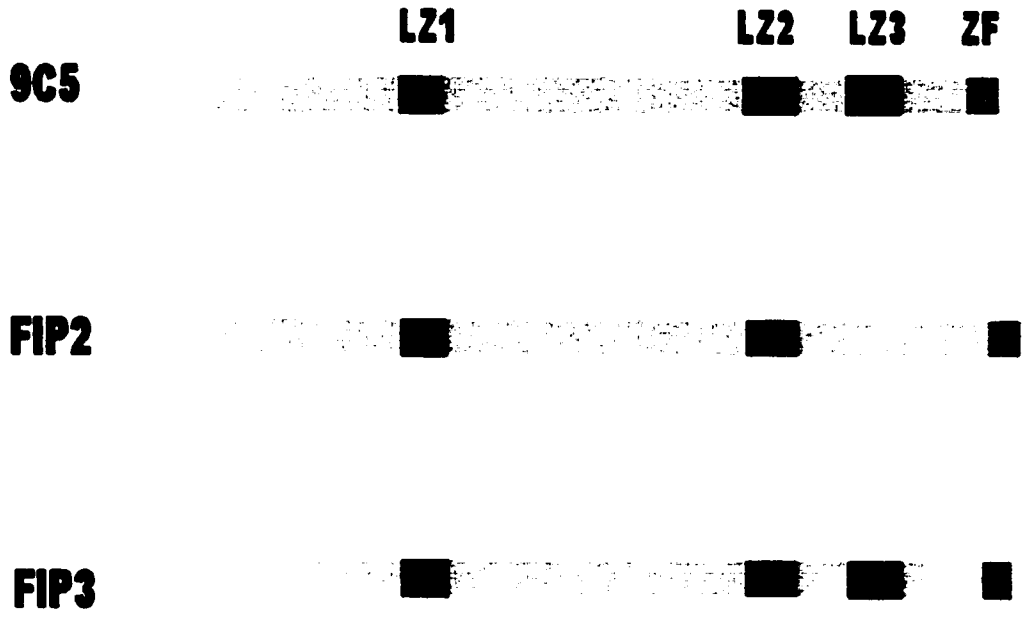


Figure 17. Structural domains of 9C5, FIP2 and FIP3.

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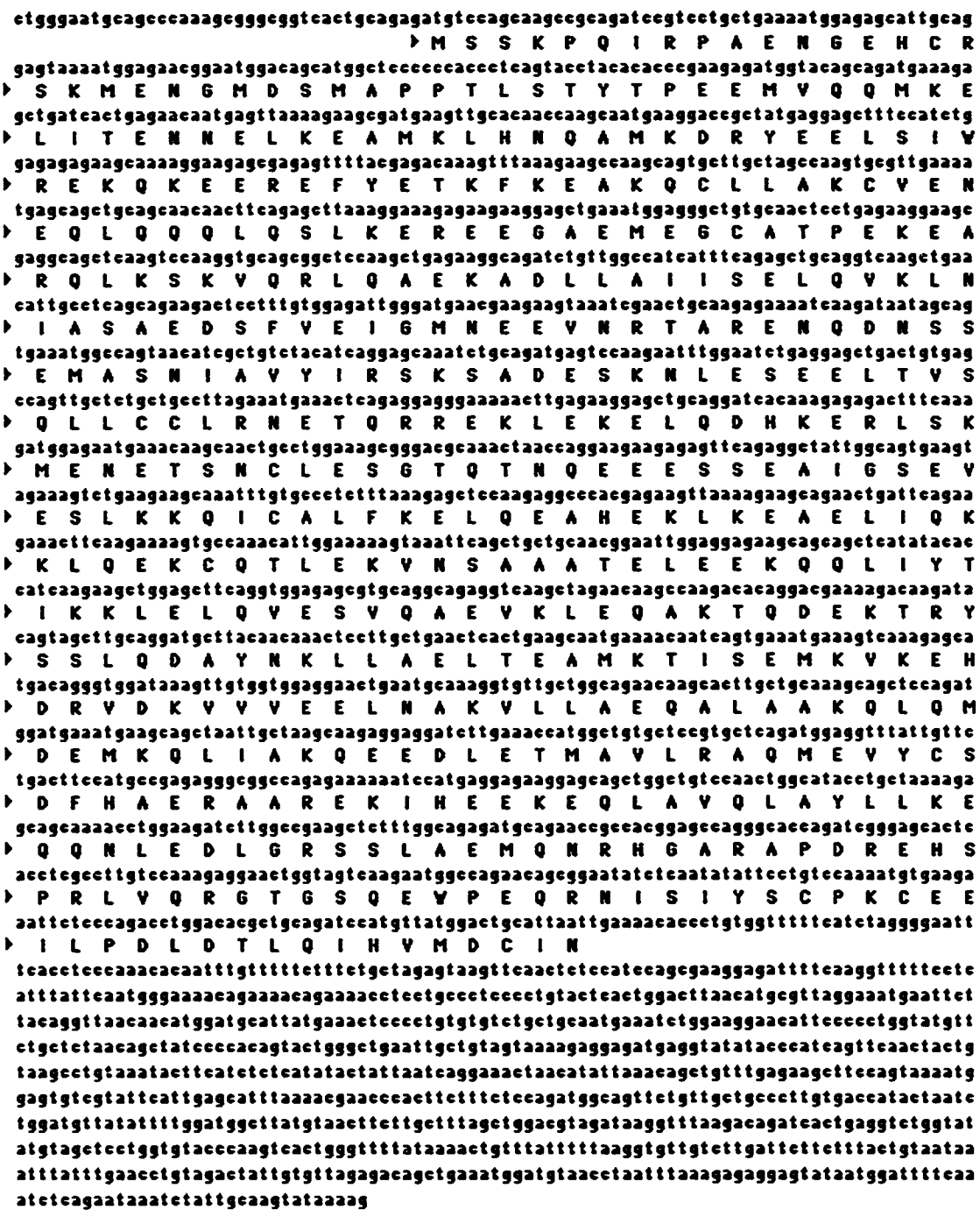

Appendix. Nucleotide sequence of 9C5. It spans 2555 basepairs and the longest ORF encodes a 556 amino acid protein. The deduced amino acid sequence is listed.